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# PERFORMANCE OF A REPETITIVE TASK BY AGED RATS LEADS TO MEDIAN NEUROPATHY AND SPINAL CORD INFLAMMATION WITH ASSOCIATED SENSORIMOTOR DECLINES

**M. B. ELLIOTT,[a] A. E. BARR,[b] B. D. CLARK,[c]**
**C. K. WADE[d] AND M. F. BARBE[e]***

[a]*Department of Neurological Surgery, Thomas Jefferson University, 909 Walnut Street, Philadelphia, PA 19107, USA*

[b]*School of Physical Therapy, College of Health Professions, Pacific University, Hillsboro, OR 97123, USA*

[c]*Department of Neurobiology and Anatomy, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129, USA*

[d]*Department of Physical Therapy, Thomas Jefferson University, Edison Building, 130 South Ninth Street, Philadelphia, PA 19107, USA*

[e]*Department of Anatomy and Cell Biology, Temple University Medical School, 3400 North Broad Street, Philadelphia, PA 19140, USA*

**Abstract—Epidemiological studies have demonstrated a relationship between advancing age and susceptibility to risk factors for median neuropathies and musculoskeletal disorders. In this study, we determined if performance of a voluntary reaching task by aged rats induced sensorimotor declines, median nerve dysfunction and increased inflammatory cytokines in peripheral nerves, muscle and spinal cord neurons. Aged (14 mon) rats were trained for 15 min/day for 4 weeks to learn a high repetition, low force (HRLF) task (19 reaches/min; 15% maximum pulling force). Aged task rats performed the task for 2 h/day, 3 days/wk, for 12 weeks (until they were 18 mon of age). No behavioral changes were detected in normal controls (NC) or food-restricted controls (FR C) as they aged. However, grip strength declined in HRLF rats in weeks 6–12 ($P<0.01$ each) and 12-week trained-only rats (TR; $P<0.05$), compared to NC. Mechanical hypersensitivity was present in weeks 9 and 12 HRLF reach limb forepaws ($P<0.01$ and $P<0.05$, respectively), and 12-week HRLF support limb forepaws ($P<0.01$) and hindpaws ($P=0.03$), compared to NC. By week 12, median nerve conduction velocity declined 23%, bilaterally, in HRLF ($P<0.001$ each), and 13% in TR ($P<0.05$), compared to NC. Tumor necrosis factor alpha (TNF$\alpha$) increased in 12-week HRLF muscle ($P=0.005$), median nerve ($P<0.01$), and neurons in superficial lamina of HRLF cervical spinal cords ($P<0.01$), compared to NC. interleukin 1 beta (IL1$\beta$) also increased in superficial lamina neurons ($P<0.01$). Loss of grip strength was correlated with median nerve conduction slowing ($r=0.70$) as well as increased nerve and muscle TNF$\alpha$ ($r=-0.38$ and $r=-0.41$, respectively); decrease in forepaw withdrawal thresholds was correlated with median nerve conduction slowing ($r=0.81$), increased nerve TNF$\alpha$ ($r=-0.59$), and increased TNF$\alpha$ and IL1$\beta$ in neurons in spinal cord dorsal horns ($r=-0.52$ and $r=-0.47$, respectively). Thus, aged rats performing a repetitive task exhibited sensorimotor declines that were associated with decreased median nerve conduction, and increased pro-inflammatory cytokines in the median nerve and cervical spinal cord neurons.** **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: aging, neuropathy, neuritis, repetitive task, work-related-musculoskeletal disorders, sensorimotor.**

Median neuropathy, such as carpal tunnel syndrome, can result from mechanical trauma such as shear or compressive forces on the nerve, particularly if repeated, and has been linked to risk factors such as gender (female), advanced age (older), and reduced fitness (Bernard, 1997; Nathan et al., 1998; de Zwart et al., 2001; Diao et al., 2005; Zambelis et al., 2010). Patients with median neuropathy report symptoms such as pain in the hands/wrists or fingers that may travel into the forearm, elbow and shoulder, as well as paresthesias, numbness and weakness (Gerr et al., 2002). An objective diagnosis of median nerve dysfunction is typically based on electrophysiological evidence of slowed median nerve conduction localized to the wrist, although the combination of electrodiagnostic findings and symptom characteristics are reported as providing the most accurate diagnosis of carpal tunnel syndrome (Rempel et al., 1999; Rempel and Diao, 2004; Diao et al., 2005).

Other risk factors for the development of neuropathies as well as several other types of musculoskeletal symptoms (MSS) and disorders (MSDs), such as radicular pain, somatic pain, myalgesias, tendinitis and tendinopathies, include performance of jobs characterized by repetitiveness, forcefulness and awkward postures (Bernard, 1997; Szabo, 1998; Gerr et al., 2002; Bonfiglioli et al., 2006, 2007; Zambelis et al., 2010). A relationship between advancing age and susceptibility to other risk factors for neuropathies and types of MSS/MSDs has also been reported (BLS, 2009; Gerr et al., 2002; Ratzlaff et al., 2007; Zambelis et al., 2010), although one longitudinal study suggests that slowing of conduction in the median nerve occurs naturally with increasing age (Nathan et al., 1998). Epidemiological data show that the incidence rate of lost workday injuries and illnesses due to repetitive motion is 1.6 times higher in workers aged 55–64 compared to those aged 25–34 (BLS, 2009). Computer operators over age 30 show increasing risk of developing neck, shoulder, arm and hand symptoms, such as pain, aching, burning, numbness or tingling, in a 3-year prospective study of MSS/MSD incidence in newly hired workers in computer intensive jobs, with the most common disorder being somatic pain syndrome (Gerr et al., 2002). Our laboratory has reported that patients with upper extremity MSS/MSDs have in-

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*Corresponding author. Tel: +1-215-707-6422; fax: +1-215-707-2966.
E-mail address: mary.barbe@temple.edu (M. F. Barbe).
*Abbreviations:* FR C, food-restricted controls; HRLF, high repetition low force; MPF, maximum voluntary pulling force; MSDs, musculoskeletal disorders; MSS, musculoskeletal symptoms; TR, trained-only rats.

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creased frequency of local signs of pain and tenderness, peripheral nerve irritation and weakness as well as increased frequency of these symptoms at multiple anatomical sites (mean age=45; range of 19–74, with 23 of 31 subjects over 30), findings that interestingly correlated with increased serum inflammatory cytokines (Carp et al., 2007).

Recent work in animal models suggests that performance of repetitive tasks induces median neuropathies, hand movement dysfunctions, and inflammatory tendinopathies (Topp and Byl, 1999; Barbe et al., 2003; Clark et al., 2003, 2004; Perry et al., 2005; Sommerich et al., 2007; Coq et al., 2009; Fedorczyk et al., 2010). Using a unique model of upper extremity MSD, we have reported that in young adult rats repetitive reaching and grasping for 8–12 weeks leads to degraded myelin, increased macrophages and cytokines, decreased nerve conduction velocity, and increased collagen deposition in the median nerve, as well as persistent inflammation in musculoskeletal tissues, woven bone formation, tendon disorganization and fibrosis, and myofiber fray (Barbe et al., 2003, 2008; Barr et al., 2004; Clark et al., 2003, 2004; Al-Shatti et al., 2005; Elliott et al., 2009b; Coq et al., 2009; Fedorczyk et al., 2010; Rani et al., 2009, 2010). These tissue changes were associated with sensorimotor declines, including reduced reach performance, decreased grip strength and changes in forepaw sensation (Barbe et al., 2003, 2008; Clark et al., 2003, 2004; Elliott et al., 2009a,b; Fedorczyk et al., 2010; Rani et al., 2010). The declines in median nerve conduction were exposure-dependent, ranging in reductions of 9–17% depending on the level of task intensity (Clark et al., 2003, 2004; Elliott et al., 2009b). We have also reported that neurochemicals involved in nociception were increased in the dorsal horns of cervical spinal cord segments with performance of repetitive tasks in young adult rats and that this increase in neurochemicals was associated with nociceptive-like behaviors (Elliott et al., 2008, 2009a,b). However, we have yet to determine if similar changes are induced in aged rats performing repetitive tasks.

Evidence that inflammatory responses in the peripheral and central nervous systems are associated with cutaneous hypersensitivity is documented in acute animal models of peripheral nerve injury (DeLeo et al., 1997; Chacur et al., 2001; Gazda et al., 2001; Milligan et al., 2003; Kelly et al., 2007). In particular, increased pro-inflammatory cytokines at the spinal cord level have been implicated in the development of cutaneous hypersensitivity in studies of cryoneurolysis, chemical insult, crush or ligature-induced chronic constriction nerve injuries in young adult rodents (DeLeo et al., 1997; Hunt et al., 2001; Winkelstein et al., 2001b; Rutkowski et al., 2002; Hubbard and Winkelstein, 2005; Svensson et al., 2005; Rothman and Winkelstein, 2007; Hatashita et al., 2008). However, an association between cutaneous hypersensitivity and a central inflammatory response has yet to be investigated in a model in which nerve dysfunction is induced by long term performance of a voluntary repetitive task.

Therefore, in this study we extended our model to aged rats performing a high repetition low force (HRLF) task. We tested the hypothesis that performance of this repetitive task by aged rats induces sensorimotor declines that are associated with peripheral nerve dysfunction and inflammation at levels similar to those observed in young rats in our previous studies (Al-Shatti et al., 2005; Clark et al., 2003, 2004; Elliott et al., 2009a). We also examined, for the first time, whether there were repetitive task-induced inflammatory changes in neurons in cervical spinal cord dorsal horns. Mechanical sensation in the hindpaws, limbs not involved in performing the repetitive task, was also examined to determine if extraterritorial cutaneous mechanical hypersensitivity was present. Moreover, since grip strength declines can be induced by intramuscular injections of pro-inflammatory cytokines (Schafers et al., 2003a; Beyreuther et al., 2007), we examined forelimb muscles involved in gripping (flexor digitorum muscles) for inflammatory cytokine levels to determine whether any task-induced increases of muscle cytokines were associated with decreases in grip strength.

## EXPERIMENTAL PROCEDURES

### Animals

All experiments were approved by the Institutional Animal Care and Use Committee in compliance with NIH guidelines for the humane care and use of laboratory animals. Studies were conducted on a total of 56 aged, female Sprague–Dawley rats (14 mon at onset of task training; 18 mon at euthanasia). Adult female rats were used for several reasons: (1) Human females have a higher incidence of work-related MSS/MSDs than males (de Zwart et al., 2001; Gerr et al., 2002; Wijnhoven et al., 2006); (2) we have used young adult female rats in extensive studies using this model, consequently our database is relevant to female rats and for comparison purposes, we prefer to continue with this gender; and (3) the examination of male rats, which are both larger and stronger, would require adjustments in operant conditioning equipment, including a switch to higher capacity force transducers, as ours were chosen for their sensitivity to the force generating capabilities of adult female rats. Rats were housed individually in the central animal facility in transparent plastic cages in a 12 h light: 12 h dark cycle with free access to water.

Thirty-eight rats were food restricted to within 5% of their naive weights. Thirty-four went through an initial training period of approximately 4 weeks, in which they were trained to perform the reaching and handle pulling task (see training regimen below). Eighteen of these trained rats then went on to perform a HRLF task (see task regimen below). The remaining 16 trained rats, serving as trained-only rats (TR), did not proceed past week 0 to the task regimen, but rested 12 weeks until euthanasia at time points matched to HRLF rats. The remaining four food restricted rats were not trained, and served as food-restricted controls (FR C). Eighteen more rats served as age-matched normal controls (NC) with free access to food. The NC rats did not undergo food restriction, training or task performance.

All rats were weighed at least weekly throughout the experiment and food adjusted accordingly. In addition to food pellet rewards, all rats received Purina rat chow daily. TR and FR C rats received daily allotments of food pellets and rat chow matched to the HRLF rats. NC had free access to food. All rats were inspected weekly and again post-mortem for presence of illness or tumors. As a consequence, an additional eight rats were eliminated from the study due to age-related health issues, such as renal failure, presence of tumors or mortality. Additional sentinel rats were examined for presence of viral infections as part of the regular veterinary care (no viruses were detected).

## Behavioral apparatus and description of HRLF task demands

The behavioral apparatus is as described in Clark et al. (2004), and depicted in Fedorczyk et al. (2010). Briefly, custom-designed force apparatuses were used (Custom Medical Research Equipment, Glendora, NJ, USA). These apparatuses were integrated into an operant behavioral training system (Med Associates, Georgia, VT, USA with Force Lever software, version 1.03.02, Med Associates). A portal was located in the wall of the operant conditioning chamber at shoulder height (3.5 cm), so the shoulder had to be fully elevated and the elbow fully extended for the animal to reach through the portal to isometrically pull a custom-designed force handle attached to a force transducer located 1.5 cm away from the portal entrance, outside the chamber wall. An auditory indicator cued the animals to reach. HRLF rats had to grasp the force handle and exert an isometric pull toward the chamber wall with a graded force effort that fell between a minimum force criterion (12.5% of maximum voluntary pulling force (MPF), determined on the last day of training using Force Lever software, version 1.03.02, Med Associates) and a maximum force criterion (17.5% MPF) for at least 50 ms. The maximum average force for this group was 34.48 g and the minimum average force was 24.63 g. If these force and time criteria were met within a 5 s cueing period, an indicator light was turned on and a 45 mg purified formula food pellet (banana flavored; Bioserve, NJ, USA) was dispensed into a trough located at floor height of the chamber in the wall panel adjacent to the aperture. To obtain the food reward, the animal had to release the handle, withdraw the forepaw from the aperture, and move to the trough to lick up the pellet.

## Training regimen–4 weeks

Prior to the initiation of the experiments, all rats were handled for 10 min/day for 2 weeks. Thirty-eight rats were food-restricted for a short period (no more than 7 days) by 5–15% of their naive weight (i.e., they lost no more than 5–15% of naive body weight) to initiate interest in the food pellets. After that first week, all rats were given extra food chow and then maintained thereafter as closely as possible to within ±5% of their naive weight until euthanasia. It is our experience that female rats require little food restriction for motivation after they have learned the task. Four of the food-restricted rats did not proceed to training, and served as FR C. Thirty-four of the food-restricted rats went through an initial training period of 10–15 min/day, 5 days/week, for approximately 4 weeks, in which they were trained to perform the reaching and handle pulling task. During this period, the rats moved through several stages of training. First, they were placed in an operant behavior box with a portal modified with an attached trough, and introduced to the banana flavored food pellets that served as food reward. When they learned to reach (without a specified reach rate) into a trough for the food pellets, a time period of typically 3–7 days, they were moved to the custom-designed operant conditioning chambers described above. In the chambers, rats learned with the aid of auditory and light cueing to reach through the portal, grasp the force handle, and exert an isometric pull on the force handle of at first approximately 1% and then 5% MPF without any specified repetition rate (1–2 weeks), and then 15% MPF without any specified repetition rate (another 1–2 weeks). By the end of this training period, rats were able to perform the HRLF task of four reaches/min at 15% MPF. Sixteen rats were randomly selected to serve as TR, and did not proceed to HRLF task performance, but rested for 12 weeks while receiving a diet similar to the HRLF rats.

## HRLF task regimen–12 weeks

At the end of the training period, 18 of the trained rats were randomly selected to begin the HRLF task regimen at the target reach rate and force requirement (four reaches/minute; 15% MPF) for 2 h/day, 3 days/wk for 12 weeks, serving as HRLF task rats. The task was divided into four, 0.5-h sessions separated by 1.5 h in order to avoid satiation. Because the inherent nature of our task is voluntary, the rats tended to over-reach, attaining an average of 19 reaches/min rather than at the target rate of four reaches/minute. In addition, they were not prevented from reaching at a higher or lower force than the target of 15% MPF. However, a food reward was not given unless they met the force criterion within a 5 s window initiated every 15 s. Rats were allowed to use their preferred limb to reach, and their contralateral limb as a support limb, as needed. The side used to reach was recorded in each session. Thus, the animals were allowed to self-regulate their participation in task performance, making this a voluntary task.

## Sensorimotor behavioral testing

The effects of the task on motor performance were evaluated bilaterally at the naive point (before food-restriction), week 0 (after training) and at the end of weeks 3, 6, 9 and 12 of task performance in age-matched HRLF rats ($n=18$), age-matched TR ($n=10$), age-matched food restricted controls (FR C; $n=4$) and age-matched NC ($n=8$). The remaining age-matched NC ($n=10$, for a total of $n=18$ NC in the study) and TR ($n=6$, for a total of $n=16$ TR in the study) rats were behaviorally tested at the naive time point and at the time of euthanasia. Grip strength of the reach and support forelimb was tested as previously described using a grip strength meter for rodents (Stoelting, Wood Dale, IL, USA) (Clark et al., 2004; Fedorczyk et al., 2010). Maximum grip strength was defined as the value of the peak force (in grams) recorded from the transducer at the moment that forepaw grip strength is overcome by the examiner. Importantly, the moment at which each animal released its grip from the handle of the grip strength meter was self-determined. Therefore, the amplitude of force generated is subject to factors such as muscle inflammation, a change that influences the behavioral performance of the animal, as described previously by Schafers et al. (2003a). The test was repeated five times per forelimb, in a randomized fashion, and the maximum grip force (strength in grams) per trial included in the statistical analysis. The person carrying out the testing was blind to treatment.

To test paw withdrawal behaviors, rats were placed into clear plastic chambers above a metal mesh ($0.5\times0.5$ cm$^2$) and acclimated for 10 min. Calibrated von Frey filaments (North Coast medical, Morgan Hill, CA, USA) were applied from below to the center of the glabrous surface of the forepaws and the hindpaws, not on the keratinized foot pads, in five applications with 10 s intervals between stimuli as described previously (Clark et al., 2004; Fedorczyk et al., 2010). The smallest force that elicited a limb withdrawal threshold was considered the threshold stimulus. Forepaw von Frey withdrawal threshold data for the preferred reach versus support limbs were kept separate for the statistical analyses. Withdrawal threshold data from right and left hindlimbs were averaged together before statistical analysis. The testing order of forepaws versus hindpaws was randomized per rat and per week. The person carrying out the testing was blind to treatment.

## Nerve conduction velocity (NCV)

In order to test focal slowing of conduction (Kimura et al., 1986; Walters and Murray, 2001), NCV was determined for the segment of the median nerve that passes beneath the transcarpal ligament. NCV was measured bilaterally in terminal surgical experiments in the median nerves of eight rats that had performed the HRLF task for 12 weeks, as well as in TR ($n=6$) and NC ($n=7$). All were 18 months of age at time of NCV testing. The method for measurement of NCV of the median nerve in rats was slightly modified from that described previously (Clark et al., 2003, 2004). Animals were deeply anesthetized using isoflurane (0.5–1.5% after induction at 4%) in air, and artificially ventilated via a tracheal cannula. Throughout surgery and recording, body temperature was maintained at 36–38 °C with a feedback-controlled heating pad, and end-tidal $CO_2$ was maintained between 30 and 40 mm Hg by adjusting ventilator settings. NCV was determined for the segment of the median nerve that passes beneath the transcarpal ligament. The median nerve was dissected free from the surrounding fascia in the forearm, and a 9 mm long cuff of polyethylene tubing supporting four silver wire leads (diameter 0.13 mm) was carefully positioned under the median nerve as it spanned through the forearm into the palm. The entire forelimb was immersed in a mineral oil bath (maintained at 36–38 °C). Stimulation was delivered via a pair of electrodes mounted into the cuff near the elbow, spaced ~1 mm apart. Cuff position was adjusted until proximal and distal monopolar recording electrodes (fixed 3.7 mm from each other) were positioned under the nerve on either side of the transcarpal ligament. Recording electrodes were referenced to a wire embedded in forearm muscles. Stimuli (10–20 $\mu$s depolarizing pulses, 5–10 V; 1.1–1.2 x threshold) were delivered at 3 Hz. For each rat, at least eight sets of averaged compound action potentials elicited from proximal and distal recording sites were digitized (Gould 6100 8-bit digital storage oscilloscope, 1 MHz/channel), with eight sweeps per average. Digitized records were lowpass filtered (87 kHz Kaiser-Bessel window). Stimulus artifacts and changing shape of the compound action potentials precluded latency estimation based on onset or peak of the waveform. Therefore, conduction latencies were calculated based on the times when the compound action potentials crossed 50% depolarization (Clark et al., 2004). NCV was calculated from the ratio of inter-electrode distance to the difference in conduction latencies at the proximal and distal recording sites. Both the surgeon and the person carrying out the recordings and data analysis were blinded to rat treatment. Tissues were not collected from rats that underwent the NCV testing to avoid confounding interpretation of results by changes induced by this surgical procedure.

## Examination of cytokines in median nerve and forelimb flexor digitorum muscles

The median nerve was examined immunohistochemically in aged HRLF task rats at 12 weeks of task performance ($n=5$), TR ($n=5$), NC ($n=6$), and FR C ($n=4$). All were 18 months of age at time of euthanasia and tissue collection. Rats were euthanized with an overdose of sodium pentobarbital (Nembutal; 120 mg/kg body weight), and were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Flexor forelimb tissues from the preferred limbs were collected as a flexor mass, postfixed "en bloc" (nerves still intact with adjacent muscle and tendon tissues) by immersion overnight, cryosectioned into 12 $\mu$m longitudinal sections, and immunostained for tumor necrosis factor alpha (TNF$\alpha$) and interleukin 1 beta (IL-1$\beta$) immunoreactive cells using tissue collection and staining methods described in Al-Shatti et al. (2005) and Elliott et al. (2008). Quantification of these cytokines in the median nerve at the level of the wrist was performed as previously described (Al-Shatti et al., 2005; Elliott et al., 2008) using an image analysis system (Bioquant, Nashville, TN, USA). The person carrying out this image analyses was blind to treatment. Adjacent sections were stained with Hematoxylin and Eosin (H&E) and examined for pathological changes such as increased collagen deposition and presence of inflammatory cells. Immunohistochemical variability was minimized by performing cytokine immunohistochemistry as a large assay in which all sections from all study groups including controls were incorporated into a single run.

Flexor digitorum muscles were collected from an additional cohort of aged HRLF rats at 12 weeks of task performance ($n=5$), TR ($n=5$), and NC ($n=5$). These tissues were collected as fresh, flash frozen tissues, homogenized and extracts assayed, in duplicate, as a batch for all study groups including controls using ELISA for levels of IL-1$\beta$ and TNF$\alpha$ using previously described methods (Barbe et al., 2008).

## Examination of cytokines in spinal cord

Spinal cords were examined immunohistochemically, bilaterally, in aged HRLF rats at 12 weeks of task performance ($n=5$), TR ($n=5$), and NC rats ($n=6$). All rats were 18 months of age at time of euthanasia. Spinal cords were collected from the above fixative perfused rats, postfixed "en bloc" by immersion overnight, cervical and upper thoracic spinal cord segments removed, and the dorsal root entry zones marked with an indelible histological ink pen (for segmental identification later in combination with Cresyl Violet morphological differences in order to distinguish C7-C8 specifically from upper cervical or thoracic spinal cord segments). Collected spinal cords segments were immersed in 30% sucrose in phosphate buffer for 3 days until cryostat sectioned into 14 $\mu$m coronal sections and placed on charged slides (Fisher Plus slides, Fisher). Spinal cord sections were blocked with 4% Blotto in 10% goat serum diluted with 0.1% Triton X-100 PBS for 1 h at room temperature. Tissues were then incubated with the following primary antibodies: NeuN antibody (a specific neuron marker; Millipore, Billerica, MA, USA; catalog no. MMB337; 1:200 dilution in PBS), TNF$\alpha$ (Millipore/Chemicon, catalog no. AB1837P; 1:250 dilution in PBS), and IL-1$\beta$ (Millipore/Chemicon, catalog no. AB1832P; 1:250 dilution in PBS), for overnight at room temperature, washed, and then incubated with appropriate secondary antibodies (all from Jackson Immuno) conjugated to Cy2 (green fluorescence) or Cy3 (red fluorescence) diluted 1:250 in PBS. Immunohistochemical variability was minimized by performing cytokine immunohistochemistry as large assays in which all sections from all study groups including controls were incorporated into no more than two runs. Numbers of Neuronal-N (NeuN) labeled neurons expressing either TNF$\alpha$ or IL-1$\beta$ were estimated in cervical superficial dorsal horns, bilaterally, using unbiased stereological counting methods in which an independent systematic sampling approach with a random start method was utilized as described by Mouton (2002). Specifically, only spinal cord levels C7-C8 were assayed in which levels were verified by (1) indelible ink markings made on cords at the time of collection, as described above, that were still visible on the fluorescence-stained spinal cord sections, and (2) examination of adjacent Cresyl Violet stained spinal cord sections in order to maintain consistency in levels counted between animals. The mean number of TNF$\alpha$+/NeuN+ or IL-1$\beta$+/NeuN+ labeled cells was counted bilaterally in spinal cord dorsal horns in superficial lamina using a 100× objective. Six measurements were made per side (both ipsilateral and contralateral to the reach arm were assayed) and per section in three cervical sections per rat; sections were separated from each other by 140 $\mu$m. Each measurement was made using a set square region of interest of 13.3 cubic microns (Bioquant, Nashville, TN, USA). Only NeuN+ labeled cells in which the nucleus was visible were measured. The person carrying out the image analyses was blind to treatment.

## Statistical analyses

To determine the effect of task performance on mechanical sensation and grip strength, two-way repeated measures ANOVAs were used with the following factors: week (naive, 0 (end of training period) and 3, 6, 9 and 12 weeks) and group (NC, FR C, TR, and HRLF). The Bonferroni post-hoc method for multiple comparisons was used to compare behavioral results in each week to naive data (within group comparisons), and to compare between groups at matching temporal end-points (inter-group comparisons); adjusted *P*-values are reported. Univariate ANOVAs were used to compare cytokines in the median nerve, flexor digitorum muscles and spinal cord, and NCVs, across groups. The Bonferroni post-hoc method for multiple comparisons was used to compare HRLF results to NC, FR C and TR results; adjusted *P*-values are reported. Spearman's nonparametric correlation tests were used to examine for associations between behavioral measures, nerve conduction velocity and tissue cytokine findings, since correlation scatter plots suggested nonlinear relationships in several cases. Data are expressed as mean±SEM.

## RESULTS

### No significant changes in weight across weeks

We first examined for changes in weight between groups across weeks, since food restriction may be one cause of sensorimotor behavioral declines. No significant differences in weight were found between groups at naive, 0, 3, 6, 9 or 12 week endpoints. For example, at the point of euthanasia (all rats were 18 mon), the mean weight per group was: NC 434±14; FR C 402±11; 12-week TR 414±16; 12-week HRLF 407±11 (in gram, mean±SEM). Correlations between weight and grip strength or mechanical sensation in either the preferred reach or support limbs at any weekly end-point were not significant, indicating no association between weight and grip strength in these rats.

### Forelimb grip strength was reduced by week 3 with HRLF task

Two-way repeated measures ANOVA showed significant differences in grip strength in reach limbs by week ($P=0.0016$) and by group ($P<0.0001$), but no significant interactions. Fig. 1A depicts significant post hoc results for the reach forelimbs and shows within group declines in grip

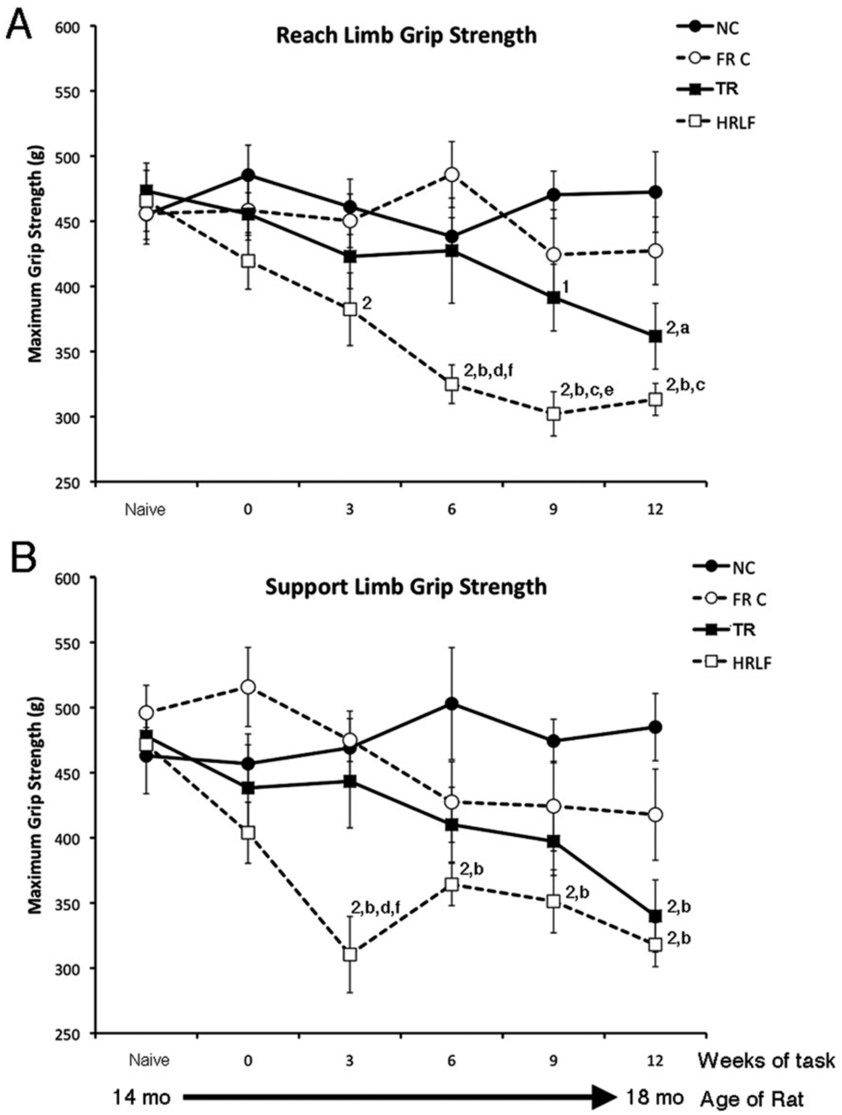

**Fig. 1.** Changes in maximum grip strength in the preferred reach limb (A) and the contralateral support limb (B) in normal controls (NC), food restricted controls (FR C), rats who trained for 4 wk and then rested for 12 wk (TR), and rats who trained and then performed a high repetition low force task (HRLF) for 12 wk. All rats were 14 mon of age at naive time point and 18 mon of age at euthanasia. $^{1}$ $P<0.05$ and 2 $P<0.01$ compared to naive data from same group, respectively; $^{a}$ $P<0.05$ and $^{b}$ $P<0.01$ compared to age-matched NC, respectively; $^{c}$ $P<0.05$ and $^{d}$ $P<0.01$ compared to age-matched FR C, respectively; $^{e}$ $P<0.05$ and $^{f}$ $P<0.01$ compared to age-matched TR, respectively.

strength in HRLF weeks 3–12 compared to naive HRLF ($P<0.01$ each), and in TR weeks 9–12 compared to naive TR ($P<0.05$ and $P<0.01$, respectively). There were also significant declines in reach limb grip strength in HRLF weeks 6, 9, and 12, compared to age-matched NC ($P<0.01$ each) and age-matched FR C ($P<0.01$, $P<0.05$ and $P<0.05$, respectively), as well as in HRLF week 6 compared to TR week 6 ($P<0.01$). Grip strength also declined in TR week 12 compared to age-matched NC ($P<0.05$).

Results for support limbs were comparable to those of reach limbs. Two-way repeated measures ANOVA showed significant differences in grip strength in support forelimbs by week ($P=0.0019$) and by group ($P<0.0001$), but no significant interactions. Fig. 1B shows within group declines in support limb grip strength of HRLF weeks 3–12 compared to naive HRLF ($P<0.01$ each), and in TR week 12 compared to naive TR ($P<0.01$). Fig. 1B depicts significant declines in support limb grip strength in HRLF weeks 3–12 compared to age-matched NC ($P<0.01$ each), in HRLF week 3 compared to age-matched FR C and TR ($P<0.01$ each), and in TR week 12 compared to age-matched NC ($P<0.01$).

No differences in grip strength were observed in NC or FR C rats as they aged from 14 to 18 months of age (Fig. 1A, B).

### Hypersensitivity is present in forepaws and hindpaws by week 12 of HRLF task

Although we have yet to examine young adult rats performing a similar level of task for changes in forepaw mechanical sensitivity, we have observed task-induced changes in withdrawal thresholds in forepaws of young adult rats performing higher demand repetitive tasks (Clark et al., 2004; Elliott et al., 2009b). Therefore, we examined aged rats for changes in forepaw sensitivity across experimental weeks and between groups. Two-way repeated measures ANOVAs showed significant differences in withdrawal thresholds in both reach and support limb forepaws by group ($P<0.0001$), but not by week. Fig. 2A shows group differences in withdrawal thresholds in the reach forepaw of HRLF week 12 compared to naive HRLF ($P<0.05$). A similar decline was observed in the support limb forepaw of HRLF week 12 compared to naive HRLF ($P<0.05$; Fig. 2B). Fig. 2A also depicts significant declines in reach limb withdrawal thresholds in HRLF week 9 compared to age-matched NC ($P<0.05$), and in HRLF week 12 compared to age-matched NC and FR C ($P<0.01$ and $P<0.05$, respectively). In the support forepaw, significant declines in withdrawal thresholds were found in HRLF week 12 compared to age-matched NC, FR C, and TR ($P<0.01$, $P<0.05$, and $P<0.01$, respectively; Fig. 2B).

We also examined hindpaws to determine if there was extraterritorial hypersensitivity as a consequence of task performance and found a significant difference by group with two-way repeated measures ANOVA ($P=0.02$). Post hoc analysis showed a decline in HRLF week 12 hindlimb withdrawal thresholds compared to naive HRLF ($P<0.05$; Fig. 2C), and compared to age-matched NC ($P=0.003$). Decreased withdrawal thresholds were observed in TR week 0 animals, but this decline did not reach statistical significance.

No differences in cutaneous sensitivity were observed in NC or FR C rat forepaws or hindpaws as they aged from 14 to 18 months of age (Fig. 2A–C).

### Reduced NCV correlates with reduced grip strength and increased forepaw withdrawal thresholds

Univariate ANOVA showed significant declines in NCV in the median nerve across groups ($P<0.001$). Post hoc analysis showed significant declines in TR week 12 (13%; $P<0.05$) and HRLF week 12, bilaterally (23%; $P<0.001$ each), compared to age-matched NC (Fig. 3A). The mean NCV was lower in HRLF week 12 reach and support limbs than for TR week 12, but the difference was not significant. Spearman's correlation showed positive associations between median NCV findings and grip strength ($r=0.70$, $P<0.0001$), and between median NCV findings and forepaw withdrawal thresholds ($r=0.81$, $P<0.0001$) (Fig. 3B, C).

### TNF$\alpha$ increased in median nerve and flexor digitorum muscle with HRLF task performance

An examination of the median nerve using immunohistochemistry showed that TNF$\alpha$ appeared to be increased in the extracellular matrix surrounding the median nerve in TR week 12 (Fig. 4B) but not within the nerve, compared to NC (Fig. 4A). In contrast, HRLF week 12 animals showed increased TNF$\alpha$ in not only inflammatory-like cells (Fig. 4C), but also in axonal profiles (Fig. 4D) and Schwann cells (Fig. 4E). Hematoxylin and Eosin stained sections revealed increased connective tissue around median nerve axon bundles (Fig. 4G), increased inflammatory cells (Fig. 4H), and axonal swellings suggestive of axonal compression (Fig. 4I) in HRLF week 12 median nerves at the level of the wrist, but not in age-matched NC (Fig. 4F). IL-1$\beta$ staining was not increased in the median nerve with training or task performance compared to NC (data not shown). In contrast, percent area fraction quantification of TNF$\alpha$ immunohistochemistry in the median nerve at the level of the wrist showed increased TNF$\alpha$ in the reach limb of HRLF week 12 compared to age-matched NC and TR ($P=0.005$; Fig. 4J). TNF$\alpha$ immunoreactivity in the median nerve was negatively correlated with grip strength ($r=-0.38$, $P=0.05$) and with forepaw withdrawal thresholds ($r=-0.59$, $P=0.002$). ELISA analysis of forelimb flexor digitorum muscles showed that IL-1$\beta$ protein levels were not significantly elevated in any group ($P=0.21$). In contrast, Fig. 4K shows that TNF$\alpha$ levels were significantly elevated in the flexor digitorum muscles of TR week 12, and HRLF week 12 reach and support limbs, compared to age-matched NC ($P<0.05$ each). TNF$\alpha$ levels in flexor digitorum muscles were negatively correlated with grip strength ($r=-0.41$, $P=0.03$), but not with forepaw withdrawal thresholds.

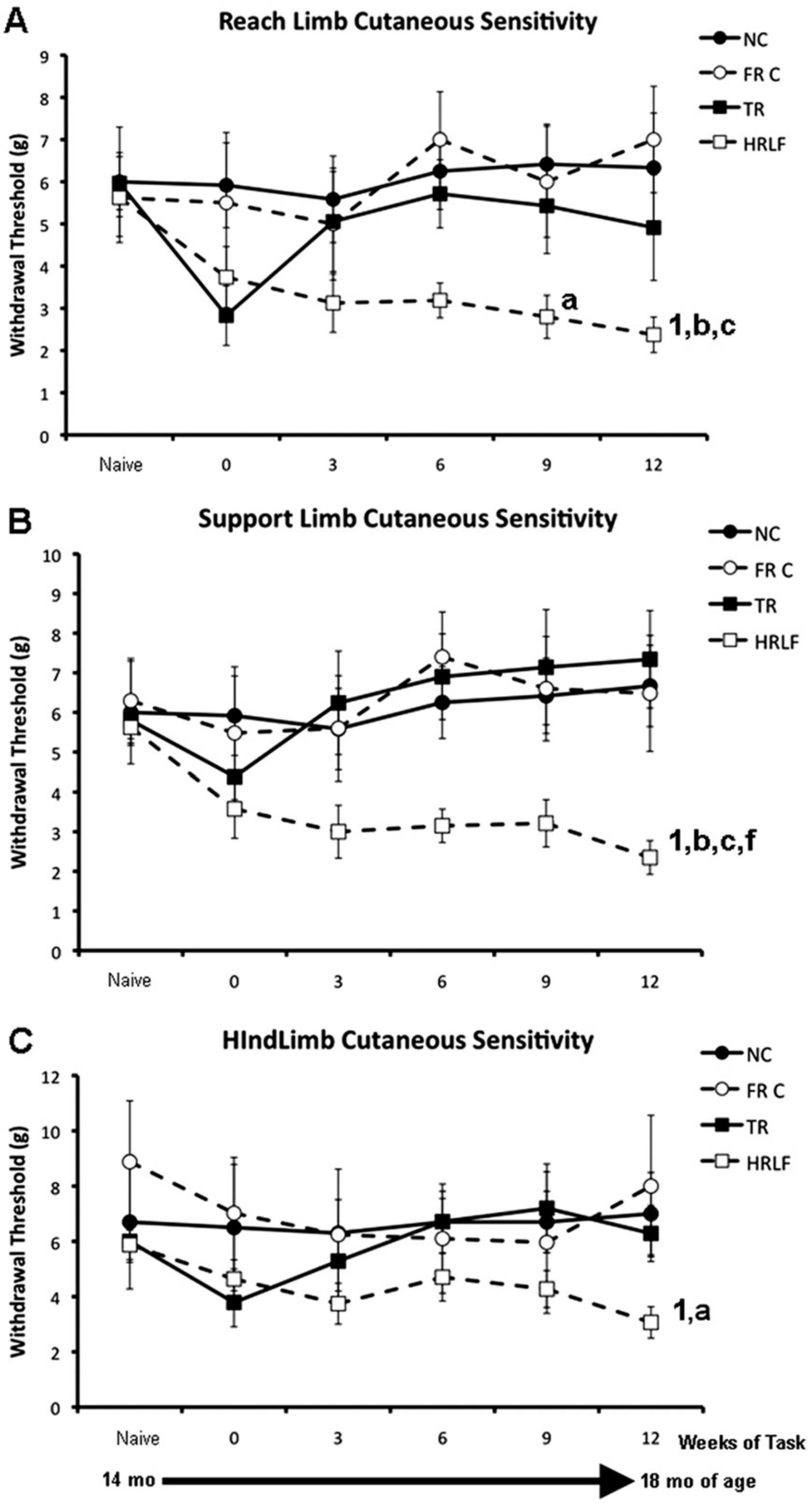

**Fig. 2.** Changes in forepaw and hindpaw withdrawal thresholds in grams (g) in normal controls (NC), food restricted controls (FR C), rats who trained for 4 wk and then rested for 12 wk (TR), and rats who trained and then performed a high repetition low force task (HRLF) for 12 wk. (A) Withdrawal thresholds in the reach limb forepaws. (B) Withdrawal thresholds in the support limb forepaws of these same rats. (C) Withdrawal thresholds in the hindpaws of these same rats. Bilateral hindpaw data was collected and averaged for each rat. [1] $P<0.05$ compared to naive data from same group; [a] $P<0.05$ and [b] $P<0.01$ compared to age-matched NC; [c] $P<0.05$ compared to age-matched FR C; [f] $P<0.01$ compared to age-matched TR.

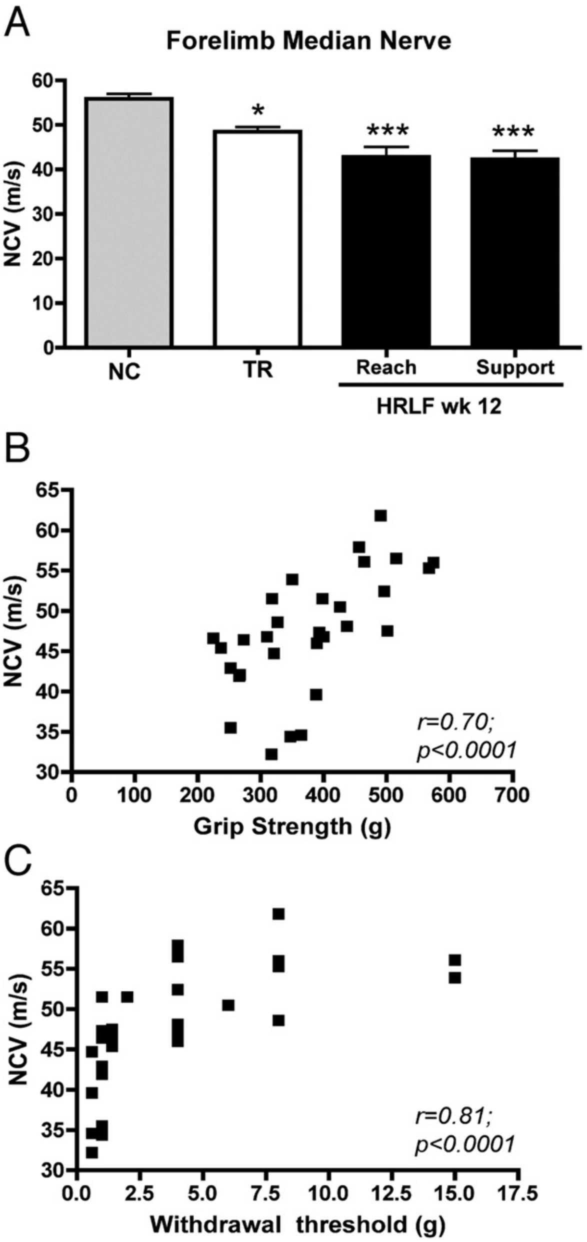

**Fig. 3.** Changes in median nerve conduction velocity (NCV) at the level of the wrist in aged normal control rats (NC), trained rats (TR) following a 12 wk cessation of training, and rats performing a high repetition, low force (HRLF) reaching and handle pulling task for 12 wk. (A) NCV of the median nerve at the level of the carpal tunnel. Bilateral data for TR is shown combined; HRLF data for preferred reach and support limbs is shown separately. * $P<0.05$ and *** $P<0.001$ compared to NC rat data. (B) Scatter plot showing positive correlation between NCV and grip strength by Spearman's Rank Correlation. (C) Scatter plot showing positive correlation between NCV and von Frey withdrawal thresholds by Spearman's *r*-test.

### TNF$\alpha$ and IL-1$\beta$ increase in spinal cord neurons with task performance

We have previously determined that rats perform this task as a bilateral task and exhibit inflammatory peripheral tissue changes bilaterally (see Barbe et al., 2003, 2008; Fedorczyk et al., 2010). In line with this observation, in the present study, no side-to-side differences were observed in dorsal horn neuronal expression of cytokines ($P>0.05$ for each cytokine assayed). In light of this, dorsal horn neuronal counts from the ipsilateral (reach limb side) and contralateral (support limb side) were combined for further statistical analyses. In HRLF week 12, we observed an increase of IL-1$\beta$ and TNF$\alpha$ cells in the dorsal horn superficial lamina that were also immunolabeled for NeuN (Fig. 5B–D and Fig. 5F–L, respectively), compared to NC (Fig. 5A and E, respectively). The number of NeuN+/IL-1$\beta$+ cells in the dorsal horns of cervical spinal cord segments of HRLF week 12 were increased compared to NC and TR ($P<0.001$ and $P<0.01$, respectively; Fig. 6A), as were the number of NeuN+/TNF$\alpha$+ cells in HRLF week 12, compared to NC and TR ($P<0.001$ each; Fig. 6B). We also observed that some cells that expressed TNF$\alpha$ or IL-1$\beta$ were not labeled for NeuN, and therefore were most likely glial cells (see cells indicated by small arrows in Fig. 5J–L; double labeled neurons are indicated with arrowheads in these same panels). The number of NeuN+/IL-1$\beta$+ immunoreactive cells in the dorsal horns was negatively correlated with forelimb withdrawal thresholds ($r=-0.51$, $P=0.009$), as was the number of NeuN+/TNF$\alpha$+ immunoreactive cells ($r=-0.47$, $P=0.01$).

## DISCUSSION

These results show that aging itself did not contribute to sensorimotor declines, but that performance of an HRLF task by aged rats was associated with grip strength declines and forelimb mechanical hypersensitivity, compared to age-matched normal controls. These behavioral changes were associated with task-induced declines in median NCV and local tissue (muscle and nerve) increases in inflammatory cytokines. Training to perform the HRLF task was also associated with grip strength and median NCV declines, compared to age-matched normal controls. We also observed, for the first time, that performance of repetitive tasks leads to increased pro-inflammatory cytokines in spinal cord neurons as well as declines in withdrawal thresholds in hindpaws, limbs not involved in performing the task.

### Effects of training and age

Our normal and food restricted control data suggest that the aging process itself with or without food restriction from 14 to 18 months was not associated with any behavioral or tissue changes. This result differs from findings by Nathan and colleagues (1998) suggesting that slowing of conduction in the median nerve occurs naturally with increasing age. Perhaps our time frame of comparison (14 months to 18 months of age) was not enough to detect any significant effects of aging in control rats. What we did observe were greater than expected declines in median nerve conduction velocity in the aged TR rats (13%) and aged HRLF task rats (23%) compared to our previously reported 9% decline in NCV in young adult rats performing a similar level task (Clark et al., 2003). Also, the 23% decline of NCV in aged HRLF rats was greater than that observed in

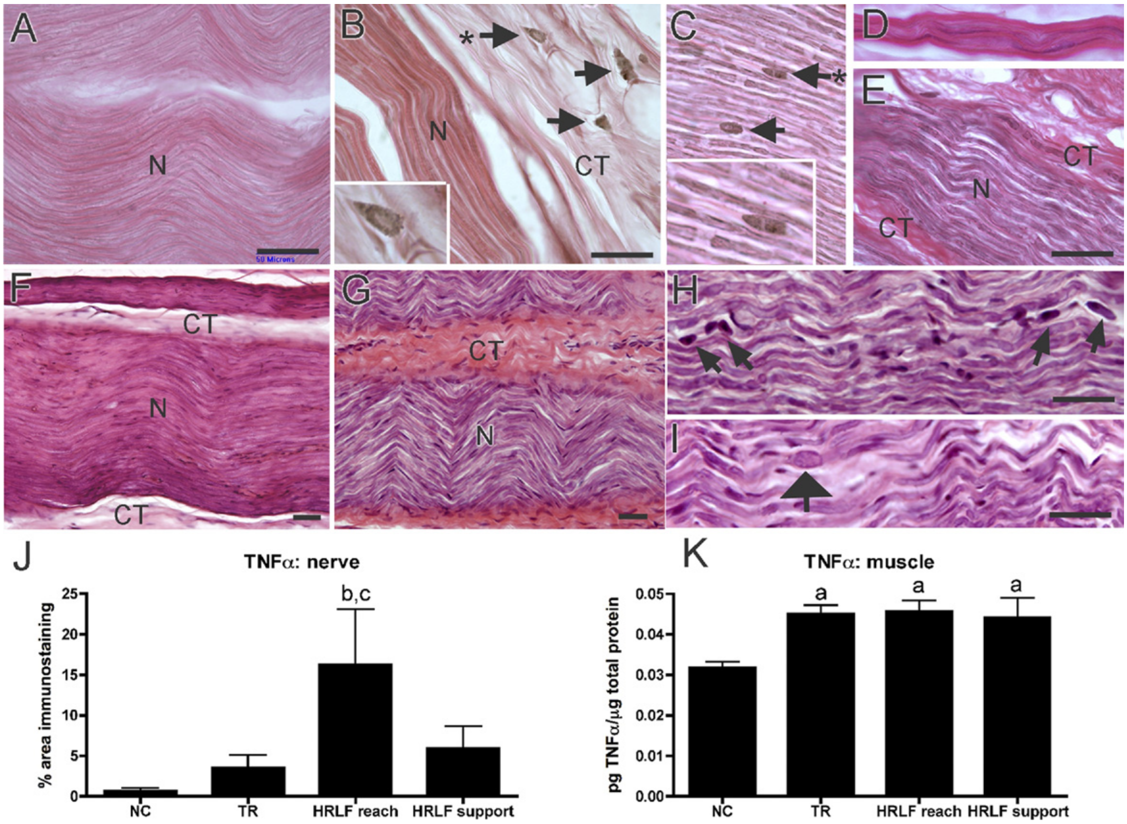

**Fig. 4.** Cytokine expression and morphology of the median nerve at the level of the wrist and flexor digitorum muscle in aged normal control rats (NC), trained rats (TR), and rats performing a high repetition, low force (HRLF) reaching and handle pulling task for 12 wk. (A–E) Immunohistochemical detection of TNFα (black staining) and Eosin counterstain (pink). (A) NC nerve (N) showing no staining for TNFα. (B) TR wk 12 showing increased TNFα immunoreactive cells (arrows) in connective tissue (CT) surrounding the nerve but not in the nerve. Inset shows higher power of cell indicated by *. (C) TNFα in inflammatory cells (arrows) and Schwann cells (myelin sheath) of a HRLF wk 12 nerve. Inset shows higher power of cell indicated by *. (D) TNFα in axonal like profiles in a small nerve in the palmar extracellular tissues. (E) TNFα in Schwann cells (myelin sheath) of a HRLF wk 12 nerve. (F–I) Hematoxylin and Eosin stained nerves. (F) NC nerve. (G) HRLF wk 12 nerve showing increased connective tissues (CT) around nerve bundles. (H) HRLF wk 12 nerve showing increased inflammatory cells (small arrows) in nerve. (I) HRLF wk 12 median nerve showing axonal swelling (large arrow). (J) Quantification of TNFα immunohistochemistry in median nerve. (K) ELISA detected levels of TNFα in flexor digitorum muscle. [a] $P<0.05$ compared to NC; [b] $P<0.01$ compared to NC; [c] $P<0.05$ compared to TR. Scale bars=50 microns. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

two prior studies from our laboratory in which young adult rats performing higher demand tasks of moderate repetition high force or high repetition high force had 15% and 17% declines, respectively, in median NCV (Clark et al., 2004; Elliott et al., 2009a). We also observed that the aged TR rats, rats who learned to perform the task during an initial 4 week period of 10 min/day of increasing task requirement until the HRLF task level was reached, showed declines in grip strength and increases in TNFα in flexor digitorum muscles. We did not observe differences in these two variables in TR compared to NC rats in a previous study from our laboratory examining young adult rats performing a similar task (Barbe et al., 2008). Since other studies have suggested a relationship between advancing age and susceptibility to other risk factors for MSS/MSDs (Gerr et al., 2002; Ratzlaff et al., 2007; Zambelis et al., 2010), and since performance of repetitive jobs is one of those other risk factors (Bernard, 1997; Szabo, 1998; Gerr et al., 2002; Bonfiglioli et al., 2006, 2007; Zambelis et al., 2010), we suggest that the aged rats in this study had increased susceptibility to median neuropathy with training and task performance perhaps due to decreased or slowed repair after the onset of tissue changes. We are currently investigating this hypothesis further.

## Signs of inflammation-linked peripheral sensitization

Our findings of mechanical hypersensitivity in the presence of decreased NCV, and histological findings of increased extraneuronal connective tissue and axonal swelling in the median nerve, are suggestive of nerve compression. These findings agree with several other clinical carpal tunnel syndrome and animal model studies of acute nerve compression. For example, hand and arm pain in the distribution of the median nerve is a common symptom in patients with electrophysiologicaly diagnosed carpal tunnel syndrome, particularly in those subjects involved in full time intensive manual work (Bonfiglioli et al., 2007). Studies examining the effects of chronic constriction injury from

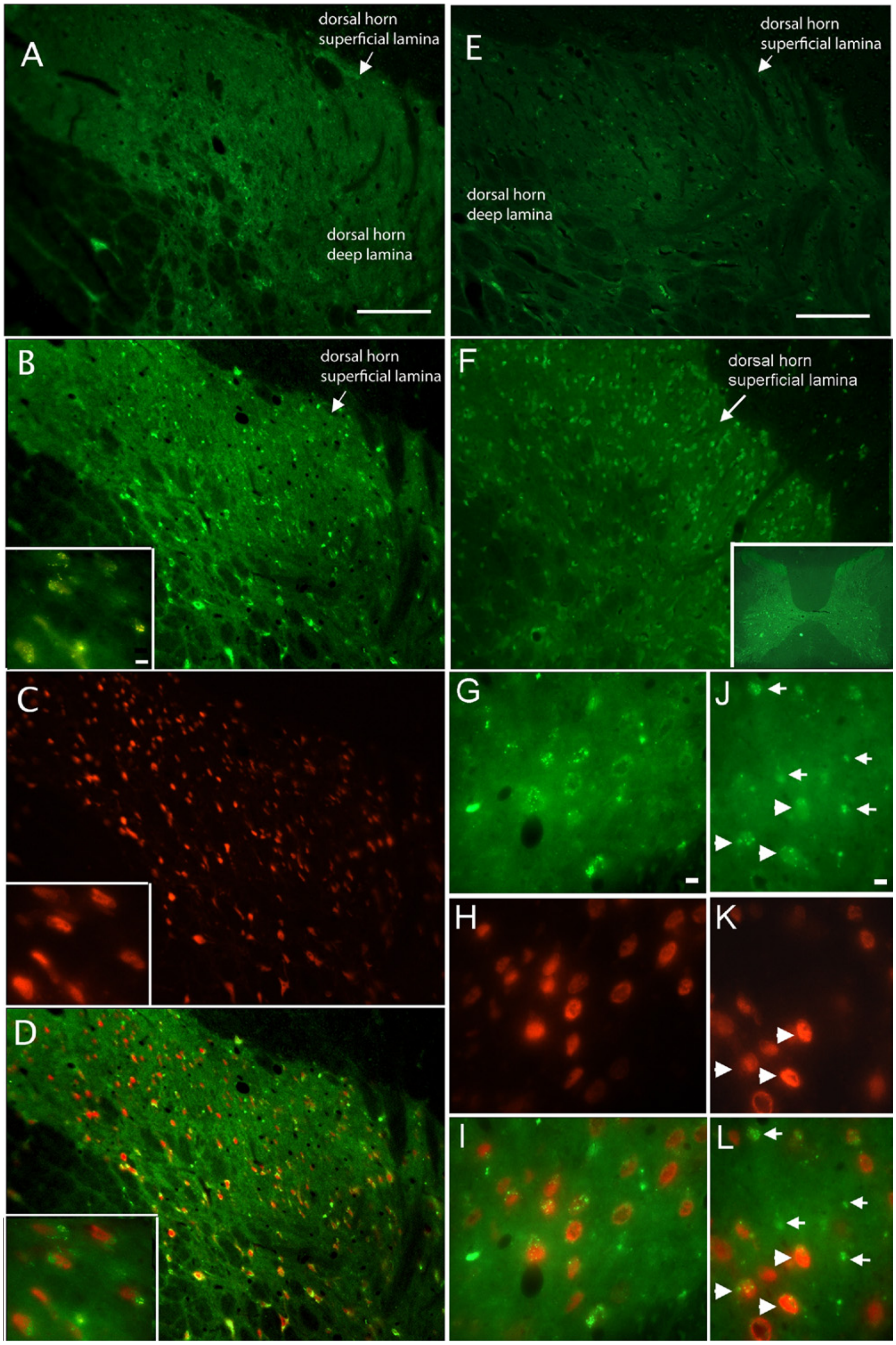

**Fig. 5.** Cytokine expression in cervical spinal cord dorsal horn neurons. (A) Normal control rat dorsal horn labeled with IL-1$\beta$ (green). (B) HRLF wk 12 rat dorsal horn labeled with IL-1$\beta$ (green); higher power of IL-1$\beta$+ cells in dorsal horn shown in inset. (C) Same section as shown in (B) labeled with Neuronal N (NeuN; red); higher power of NeuN+ cells from same site as inset in (B) shown in inset. (D) Merged (B) and (C); inset shows several cells that are double labeled for both NeuN and IL-1$\beta$. (E) Normal control rat dorsal horn labeled with TNF$\alpha$ (green). (F) HRLF wk 12 rat dorsal horn labeled for TNF$\alpha$ (green). Inset shows low power photo of cervical cord. (G) Higher power photomicrograph of HRLF wk 12 rat dorsal horn labeled for TNF$\alpha$. (H) Same section as (G) shown labeled with NeuN. (I) Merged (G) and (H) showing several cells that are double labeled for both NeuN and TNF$\alpha$. (J) Higher power photomicrograph of HRLF wk 12 rat dorsal horn labeled for TNF$\alpha$. (K) Same section as (J) shown labeled with NeuN. (L) Merged (J) and (K). Arrowheads in (J–L) indicate cells expressing TNF$\alpha$ that also express NeuN; small arrows indicate cells expressing TNF$\alpha$ that do not express NeuN. Scale bars in (A) and (E)=25 microns. Scale bars in (B) inset, (G), and (J)=10 microns. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

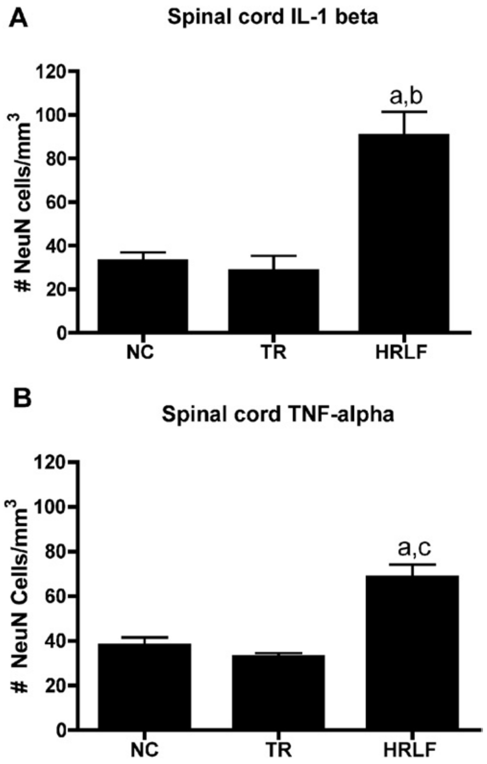

**Fig. 6.** Number of neurons labeled with Neuronal N (NeuN) antibody that co-express (A) IL-1$\beta$ and (B) TNF$\alpha$ in the spinal cord dorsal horn superficial lamina. $^a$ $P<0.01$ compared to normal controls (NC); $^b$ $P<0.01$ compared to trained controls (TR). HRLF, rats performing a high repetition, low force (HRLF) reaching and handle pulling task for 12 wk.

nerve ligation also consistently report mechanical hypersensitivity (Winkelstein et al., 2001b; Schafers et al., 2003b; Svensson et al., 2005).

Our observed mechanical hypersensitivity was also associated with an increase of TNF$\alpha$ in the median nerve. Pro-inflammatory cytokines have been shown to sensitize peripheral terminals of nociceptors both directly and indirectly, leading to hypersensitivity (Moalem and Tracey, 2006; Schafers and Sorkin, 2008). We have previously reported forepaw mechanical hypersensitivity in young adult rats performing a moderate repetition high force task for 12 weeks coincident with inflammatory responses in forelimb musculoskeletal and nerve tissues (Elliott et al., 2009b). The present finding of mechanical hypersensitivity in both forepaws is likely due to the bilateral nature of the task, in which non-reaching limbs are used to push against the wall of the behavioral chambers for support (Fedorczyk et al., 2010). Thus, the bilateral hypersensitivity responses in our study are not a type of “mirror allodynia” sometimes seen after unilateral nerve ligation, in which there is a contralateral spread of symptoms via spinal cord mechanisms (DeLeo et al., 1997; Chacur et al., 2001; Milligan et al., 2003; Kelly et al., 2007), but rather due to bilateral use of the forelimbs in performing the task, and then bilateral changes in the median nerves.

Similar to the current findings, we have previously observed task-induced grip strength declines in young adult rats performing a similar demand repetitive task (Barbe et al., 2008). The contribution of nerve dysfunction and/or nerve inflammation to motor declines in our model is supported by the correlations between forelimb grip strength and both median nerve conduction velocity and inflammatory cytokine levels. However, forelimb grip strength declines can also occur after intramuscular injections of TNF$\alpha$ into forelimb muscles (Schafers et al., 2003a; Beyreuther et al., 2007). In a recent study from our lab, a two-week regimen of anti-TNF$\alpha$ drug decreased repetitive task-induced increases of TNF$\alpha$ in flexor forelimb muscles and attenuated the declines in grip strength (Rani et al., 2010). These findings, combined with our current findings of increased muscle TNF$\alpha$ and their statistical association with behavioral declines are suggestive of inflammation-driven peripheral sensitization contributing to sensorimotor changes with performance of repetitive tasks.

### Signs of inflammation-linked central sensitization

Hindpaw mechanical hypersensitivity in the present study is suggestive of an extraterritorial spread of symptoms since the hindpaws were not used to perform this upper extremity task. Studies showing mirror allodynia or extraterritorial pain in cases of unilateral inflammatory neuritis provide evidence for mechanisms of central sensitization (Chacur et al., 2001; Gazda et al., 2001). The phenomenon of central sensitization is characterized by adaptations such as changes in neuronal structure, protein production, function, and survival within the CNS that then contribute to abnormal pain behavior, as well as altered biochemical and cellular responses (Woolf and Salter, 2000). It has been proposed that spinal cord cytokines released in the dorsal horn terminal region ipsilateral to the affected peripheral nerve spread to nearby nerve terminals, affecting other nerves and sensory processing, and in turn producing remote and contralateral effects (Chacur et al., 2001). Unfortunately, we did not collect lumbar spinal cord segments, and therefore are unable to determine if cytokines also increased in lumbar spinal cord segments. Despite this limitation of our study, the finding of mechanical hypersensitivity in body regions not involved in performing the task is suggestive of central mechanisms of sensitivity and is of potential interest to clinicians considering appropriate therapies for patients with MSS/MSDs. Alternatively, a task-induced systemic cytokine response may also be associated with the widespread mechanical hypersensitivity found in the present study; we have previously observed a significant correlation between reduced grip strength and task-induced increases in serum inflammatory cytokines (Barbe et al., 2008; Elliott et al., 2009a,b).

Because inflammatory cytokines increased in both the median nerve and in spinal cord neurons as a consequence of task performance, we cannot separate peripheral versus central inflammatory mechanisms contributing to the observed cutaneous sensation changes in the forepaws. We can only point to an abundance of other studies showing spinal cord inflammatory responses after unilateral peripheral nerve injury, for example increased activated microglia and spinal cord neuron- and glia-produced cytokines, increases that are temporally associated with mechanical hypersensitivity (DeLeo et al., 1997; Hunt et al., 2001; Shubayev and Myers, 2002; Schafers et al., 2003b; Ohtori et al., 2004; Hubbard and Winkelstein, 2005; Hatashita et al., 2008). For example, the pro-inflammatory cytokines TNF$\alpha$ and IL-1$\beta$, significantly increased in the spinal cord in two models of mononeuropathy, chronic constriction and cryoneurolysis of the sciatic nerve (DeLeo et al., 1997). The contribution of central sensitization to MSD-induced cutaneous hypersensitivity is also supported by past findings from our laboratory showing increased levels of substance P and neurokinin-1 receptors in the dorsal horns of cervical spinal cord segments of young adult rats performing a similar repetitive task (Elliott et al., 2008, 2009a,b).

We examined spinal cord dorsal horns for only neuronal-produced inflammatory cytokines. This is another limitation of our study. Although neurons are known to be one cellular source for cytokines within the CNS (DeLeo et al., 1997; Schafers et al., 2002; Shubayev and Myers, 2002), many studies have focused on glial cell production of inflammatory cytokines in the spinal cord after peripheral nerve injury (Winkelstein et al., 2001a; Milligan et al., 2003; Hatashita et al., 2008). We present evidence that the production of cytokines in the spinal cord in our model includes neurons. That said, the production of cytokines by glial cells is also clearly plausible (see Fig. 5) and still needs to be investigated in our model for full interpretation and understanding of the central changes induced by performance of this voluntary repetitive task.

## CONCLUSION

Our study shows that performance of a repetitive reaching and grasping task by aged rats resulted in sensorimotor declines, slowed conduction velocity in the median nerve, and increased pro-inflammatory cytokines in peripheral nerve, muscle and spinal cord neurons. We also show an association between the sensorimotor declines and several of the tissue changes. Our findings further suggest that both peripheral and central sensitization mechanisms may contribute to sensorimotor declines, although we have only examined spinal cord neuronal involvement to date. However, the aging process alone across the duration of the study (14 to 18 mon of age in Sprague–Dawley rats) was not associated with behavioral declines or tissue changes.

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