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Spinal substance P and neurokinin-1 increase with high repetition reaching

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ABSTRACT

Musculoskeletal injury and inflammation is associated with performance of repetitive and forceful tasks. In this study, we examined the effects of performing a voluntary, highly repetitive, negligible force (HRNF) reaching task on spinal cord neurochemicals involved in nociception. To our knowledge, no other laboratories are examining spinal cord nociceptive neurochemicals in response to repetitive motion-induced injury and inflammation. The purpose of this study was to extend our earlier findings related to central neurotransmitters from a low demand task to a higher demand task. Specifically, this study determined immunoreactivity of a peptidergic pro-nociceptive transmitter (substance P) and one of its receptors, neurokinin-1 (NK-1) receptor, in spinal cord dorsal horns in rats performing a HRNF reaching task for 6–10 weeks. The relationship of these spinal cord changes with the number of TNFα immunopositive cells in flexor forelimb muscles and with previously observed forearm grip strength changes from these same rats were examined. Performance of the HRNF task resulted in significantly increased substance P and NK-1 receptor immunoreactivity in the superficial lamina of spinal cord dorsal horns at 6 and 10 weeks compared to trained controls ($p < 0.01$). The increased substance P and NK-1 receptor immmunoreactivity were positively correlated with declines in forearm grip strength, an assay of movement-related hyperalgesia ($r = 0.70$, $p < 0.01$ and $r = 0.64$, $p < 0.05$, respectively). The increased substance P and NK-1 receptor immmunoreactivity were also positively correlated with increased TNF immunopositive cells in forelimb flexor muscles ($r = 0.85$, $p < 0.001$ and $r = 0.88$, $p < 0.001$, respectively). Thus, our highly repetitive task leads to increased spinal cord pro-nociceptive neurochemicals that are most likely directed by forelimb muscle inflammation and pain.

Epidemiological evidence supports that musculoskeletal injury and inflammation of the upper extremity is associated with the performance of repetitive and forceful tasks [6]. According to the Bureau of Labor Statistics, repetitive motion such as typing and repeated grasping was the exposure that resulted in the longest absences from work in 2005 and 2006 [8,9]. More than 5 h a week in productive time are lost in workers due to common pain conditions such as back pain, arthritis, and musculoskeletal pain, costing an estimated $61.2 billion annually [35].

A number of investigations have found histopathological changes following repetitive motion tasks, including necrotic muscle fibers with inflammatory cell infiltrates, and increased pro-inflammatory cytokines in musculoskeletal tissues [1–4,10–16,24,26,30,31,34]. A small number of studies investigating repetitive motion injuries have shown that peripheral nerve and musculoskeletal inflammation is accompanied by decreased grip strength and other motor performance declines [10,11,33]. Reduced grip strength in association with muscle inflammation has been proposed as a sign of movement-related or muscle hyperalgesia [7,19,20,36,38]. Several studies have shown that peripheral inflammation increases afferent nociceptor terminal sensitivity and excitability [17]. At the spinal cord level, this sensitization may cause increased neurotransmitter, receptor, or ion channel expression [41] and is a proposed mechanism of chronic pain [40]. To our knowledge, no other laboratories are examining spinal cord neurochemicals related to nociception in response to a repetitive motion-induced musculoskeletal inflammation and movement-related pain.

To date, studies from our laboratory show that voluntary performance of a high repetition, negligible force (HRNF; mean reach rate of 8 reaches/min; <5% maximum voluntary pulling force) upper extremity reaching and food retrieval task for 3 months resulted in local nerve and musculoskeletal inflammatory responses, increased serum levels of pro-inflammatory cytokines and chemokines, and

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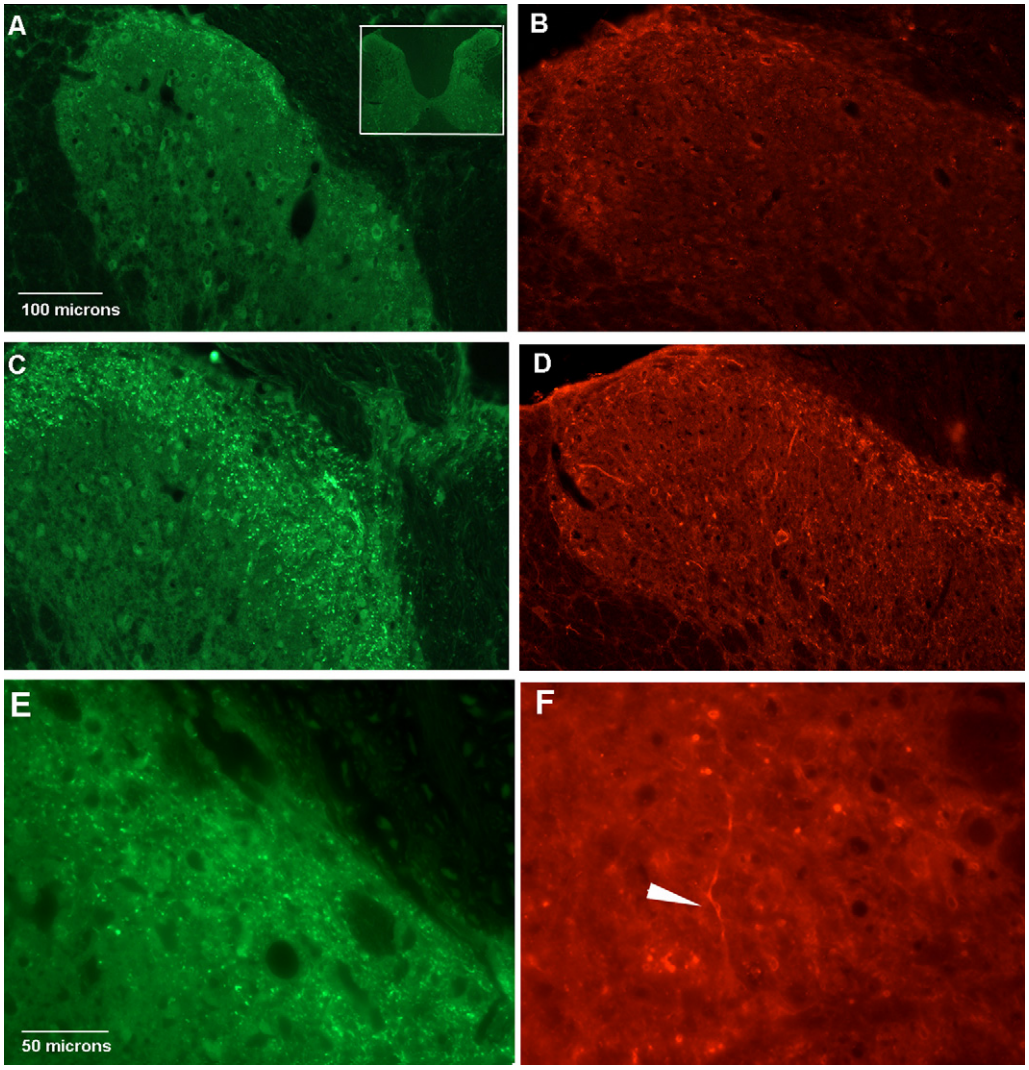

Fig. 1. Substance P and neurokinin-1 immunostaining in the cervical spinal cord dorsal horn superficial lamina. (A) Control rats have lower levels of substance P immunofluorescence staining in the superficial lamina of the dorsal horns compared to panel C. Inset in panel (A) shows a C7 spinal cord cross-section at low power. (B) Control rats have lower levels of neurokinin-1 immunofluorescence staining in the dorsal horn superficial lamina compared to panel D. (C) High levels of punctuate substance P immunofluorescence (green) staining is present in the dorsal horns of HRNF rats at week 6. The substance P immunostaining is distributed across the entire zone, medial (right side of panel) to lateral, of the superficial lamina with increased expression laterally. (D) Neurokinin-1 receptor immunofluorescence (red) staining spans the entire zone of the superficial lamina of HRNF rats at 6 weeks. Increased neurokinin-1 expression is observed more medially. (E) Punctate appearance of substance P shown under high power in dorsal horn superficial lamina in HRNF week 6 rats. (F) Neurokinin-1 staining shown under high power appears on soma plasma membranes and neuronal process-like structures (white arrow head) in HRNF week 6 rats. A–D scale bars = 100 μm; E and F scale bars = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

declines in sensory and motor function [1,3–5,11]. Recently, we explored the effects of a low repetition, negligible force (LRNF; mean reach rate of 3 reaches/min) task and found mild inflammatory responses in nerve and bone, increased neurochemicals (substance P and neurokinin-1) in spinal cord dorsal horns after 8 weeks, and losses in fine motor control, although gross motor function was preserved [13]. However, the spinal cord responses to our HRNF task, a higher demand task, have yet to be examined. Therefore, the purpose of this study was to extend our earlier findings related to central neurotransmitters from our study of a low demand task [13] to a higher exposure task. Specifically, we determined immunoreactivity of a peptidergic pro-nociceptive transmitter (substance P) and one of its receptors, neurokinin-1 (NK-1) receptor, in spinal cord dorsal horns in rats performing a HRNF reaching and food retrieval task for 6 or 10 weeks. This study centers around the hypothesis that increased spinal substance P and neurokinin-1 receptor immunoreactivity is a result of peripheral inflammation in musculoskeletal tissues. The relationship of spinal cord changes with the number of TNFα immunopositive cells in flexor muscles and with previously observed forelimb grip strength test results (data published in Barbe et al., 2008) [4] from these same rats were examined.

Adult female Sprague–Dawley rats (3.5 months of age at onset of experiments) were obtained from ACE, PA. The animals were housed in the Central Animal Facility on the Health Science Campus at Temple University. Animal care and use was monitored by the University Animal Care and Use Committee to assure compliance with the provisions of Federal Regulations and the NIH "Guide for the Care and Use of Laboratory Animals". Rats were randomized into one of two groups: HRNF or trained control. Rats were trained to perform the HRNF task previously described in detail [3,11] for periods of 6 weeks ($n = 5$) or 10 weeks ($n = 4$). The experimental ($n = 9$) and trained control ($n = 5$) rats learned the task during an initial 10–12 day training period. When they were able to perform the task consistently, the experimental animals began performing the HRNF task regimen of 4 half hour training sessions separated

by 1.5 h at the defined target rate of 4 reaches/min for 2 h/day, 3 days/week for 6–10 weeks. The rats tended to overreach, making the actual reach rate an average of 8 reaches/min.

Animals were euthanized by lethal overdose of Nembutal, 120 mg/kg body weight (consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association) and perfusion fixed with 4% paraformaldehyde. The cervical region of spinal cords were removed, postfixed "en bloc" by immersion overnight, equilibrated for 3 days in 30% sucrose (at 4 °C), cryosectioned into 13 μm coronal sections, and mounted onto coated slides (Ultrastick; Corning).

Immunohistochemical analysis was performed using previously described methods [1,13], in which the individual performing of the immunohistochemical analysis was blinded to the groups. Briefly, spinal cord sections were blocked with goat serum and incubated with rabbit anti-substance P antibody (1:250 in phosphate buffered saline (PBS), Chemicon, catalog no. AB1566), and rabbit anti-NK-1 receptor antibody (1:1000 in PBS, Chemicon, catalog no. AB5060) for overnight at room temperature, and then appropriate secondary antibodies (1:100 in PBS; Jackson Immuno) conjugated to Cy2 (green fluorescence) or Cy3 (red fluorescence) for 2 h at room temperature. Specificity of the substance P and NK1 receptor primary antibodies used in this study has been previously confirmed in studies in which immunostaining was abolished when the antibody was preabsorbed with its respective immunogen, substance P or NK-1 receptor [27,42]. Negative control slide staining from which either primary antibodies or secondary antibodies were omitted were also performed. To determine the changes in substance P and NK-1 receptor immunoreactive product in the spinal cord, immunofluorescent stained slides were analyzed using the videocount area and field mode options of Bioquant Osteo II as described previously [13]. Three fields were measured using a 20× objective at 700 magnification in 3 sections per spinal cord per rat (n = 4–5/group). Also, forelimb flexor muscles were collected from the same rats, sectioned longitudinally, and stained for TNFα using previously described methods [1]. Three fields were measured per muscle and per rat using previously described methods [1,3].

Univariate ANOVAs were used to determine whether week of task performance had any effect on the levels of immunoreactivity in the spinal cord dorsal horn. All statistical analyses and calculations (p-values, as well as r-squared and F-values associated with ANOVAs) were derived using Prism statistical software; a p-value of <0.05 was considered significant for all analyses. Post hoc analyses were carried out by the Bonferroni method for multiple comparisons, and adjusted p-values are reported. Pearson's correlation tests (one-tailed) were performed to examine the relationship of substance P with the number of TNF immunopositive cells in flexor muscles from the same rats and with grip strength test results from the same rats used in this study using data previously published in Barbe et al., 2008 [4].

Increased substance P and NK-1 receptor immunoreactivity in spinal cord dorsal horns of rats performing the HRNF task (Fig. 1C–F) were statistically significant compared to trained controls (Fig. 1A and B). Mean levels of substance P in spinal cord dorsal horns of trained control rats were 1.4% ± 0.6 S.E.M., while mean levels in rats performing the HRNF task were 9.0% ± 2.0 S.E.M. and 7.9% ± 1.1 S.E.M. at 6 and 10 weeks, respectively (Fig. 2A). Performance of the HRNF task resulted in significantly increased substance P immunoreactivity at 6 and 10 weeks compared to trained controls (ANOVA p = 0.004, F = 9.2, r^2 = 0.63), with the highest levels at 6 weeks (p < 0.01) (Fig. 2A). Mean NK-1 receptor levels in spinal cord dorsal horns of trained control rats were 0.7% ± 0.3 S.E.M., whereas mean levels in rats performing the HRNF task were 4.4% ± 0.7 S.E.M. at 6 weeks and 4.7% ± 1.6 S.E.M. at 10 weeks (Fig. 2B). Task performance resulted in significantly increased NK-1 receptor immunoreactivity at 6 and 10 weeks compared to trained controls (ANOVA p = 0.02, F = 6.9, r^2 = 0.67) (Fig. 2B). Performance of the HRNF task also resulted in significantly increased numbers of TNFα immunopositive cells at 6 and 10 weeks compared to trained controls (ANOVA p = 0.003, F = 11.82, r^2 = 0.72), with the highest levels at 10 weeks (p < 0.01): mean ± S.E.M. for trained controls were 0 ± 0, for HRNF 6 weeks were 6.40 ± 2.27, and for HRNF 10 weeks were 14.21 ± 3.43. The observed TNFα immunopositive cells appeared to be primarily macrophages that had infiltrated the muscles. The increased substance P and NK-1 receptor immunoreactivity in the dorsal horns were positively correlated with the number of TNFα immunopositive cells (substance P: r = 0.85, p < 0.001, r^2 = 0.72; NK-1 receptor: r = 0.88, p < 0.001, r^2 = 0.78). The increases in substance P and NK-1 receptor immunoreactivity in the dorsal horns were also positively correlated with the reduced grip strength in these same HRNF rats (substance P: r = 0.70, p < 0.01, r^2 = 0.45; NK-1 receptor: r = 0.64, p < 0.05, r^2 = 0.41). See Fig. 6 in Barbe et al. (2008) for grip strength results [4].

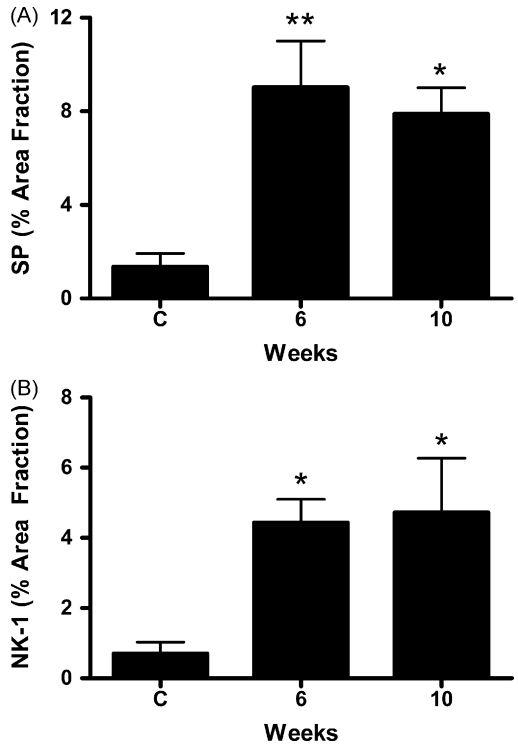

Fig. 2. Mean (+S.E.M.) percent immunofluorescent staining for substance P and neurokinin-1 (NK-1) receptor in the spinal cord dorsal horn superficial lamina. Normal and trained controls (C, n = 5) and rats that had performed the HRNF task for 6 weeks (n = 4) or 10 weeks (n = 4) were quantified. Significant increases from control levels are denoted by symbols (*p < 0.05 and **p < 0.01 compared to controls).

Substance P and its preferred receptor, neurokinin-1, were altered in spinal cord dorsal horns by continued performance of a highly repetitive task, changes that lasted for the full duration of the study. Our previous work shows that this HRNF task induced peripheral inflammation in muscle, tendon, bone, nerve and serum that matched temporally with a 9% decline in nerve conduction velocity and declines in several motor behavioral variables, including reductions in grip strength [1,3,4,11–13]. In the present study, increased substance P and neurokinin-1 receptor immunoreactivity in the spinal cord correlated strongly with declines in grip strength in these same HRNF rats. We have also observed declines in forelimb grip strength as well as forepaw cutaneous hypersensitivity in association with increased substance P and NK-1 receptor immunoreactivity in spinal cord dorsal horns in a recent study examining the effects of performing a moderate repetition high force (MRHF) task [12]. Studies by Kehl et al. suggest that reduced

grip strength is a measure of muscle hyperalgesia [18,20]. Also, declines in forelimb grip strength as an indicator of myalgia have been shown to occur after injection of TNF into forearm muscles (Beyreuther et al., 2007).

Exposure level, i.e. severity of injury, in our model affects both the temporal pattern of neurochemical changes and the temporal pattern of inflammation. Increased substance P and NK-1 receptor immunoreactivity in the spinal cord occurred in week 6 with this HRNF task, 2 weeks earlier than with our previously studied LRNF task [13]. The peak macrophage response in the median nerve of HRNF rats [11] was also 2 weeks earlier in HRNF rats than in the LRNF rats [13], as well as 4-fold greater. The macrophage and cytokine responses in musculoskeletal tissues occurred between weeks 5 and 8 in the HRNF rats, matching the timing of the spinal cord increases in substance P and NK-1 receptor in these rats [3,4]. In contrast, there were late (not occurring until week 12) increases in inflammatory cells or cytokines in musculoskeletal tissues of the LRNF rats. These findings give further support our hypothesis that timing and severity of injury/inflammation alters the temporal pattern of neurochemical changes.

Substance P, an excitatory nociceptive peptide also known to be a pro-inflammatory mediator enlists a wide array of immunomodulatory capabilities, for example, promoting the production of inflammatory mediators (prostanoids, cytokines, chemokines, nitric oxide excitatory amino acids, and ATP) from immune cells and glia that then augment neuronal excitability [22]. The pro-nociceptive actions of substance P are intertwined with glutamatergic neurotransmission in which both substance P and glutamate cause neuronal excitation, both directly and indirectly. Substance P and other tachykinins not only enhance glutamate receptor responsiveness, but also modulate pre-synaptic neurotransmission by increasing glutamate release [23]. Both substance P and glutamate activation of NK1 and NMDA receptors, respectively, results in increased calcium influx which then leads to the production of nitric oxide synthase and nitric oxide (NO) [22]. NO is thought to act as a retrograde messenger as part of a positive feedback loop leading to further increases in the release of substance P and glutamate.

The hypothesis that peripheral inflammation results in increased substance P and neurokinin-1 receptor in central afferent neuronal processes is supported by the strong positive correlation for increased substance P and neurokinin-1 receptor immunoreactivity with increased TNFα immunopositive cells. Results of several studies suggest that increased substance P in the spinal cord dorsal horns may be due to inflammation-induced increases in afferent synaptic input to the spinal cord through an increased rate of discharge, increased peptide production by the dorsal root ganglion, and/or afferent fiber phenotype alterations that favor substance P expression [25,28]. Schaible et al. described this type of afferent influx of excitatory transmitters into the spinal cord dorsal horn after injury as the pre-synaptic component of central sensitization [32], which may then contribute to abnormal pain conditions, such as hypersensitivity and chronic pain [40]. Increases in NK-1 receptor, a receptor for substance P, in the spinal cord has been proposed to occur as a response to the increased release of substance P from nociceptive afferent terminals [28]. Alternatively, spinal cord inflammatory responses to peripheral injury may create a novel environment for second order neurons and interneurons whereby they modulate synaptic function. A number of studies investigating peripheral tissue injury have shown, for example, glial activation, increased pro-inflammatory cytokines and increased expression of spinal nociceptive neuropeptides following a variety of peripheral insults including chemical, compressive and transection injuries [16,21,29,37,39]. A centrally occurring inflammatory response, such as activation of glia residing in the spinal cord, that contributes to the neurochemical changes found in the spinal cord cannot be ruled out in the present study. Studies are currently being conducted to determine whether spinal cord inflammation plays a role in the peripheral neuropathy associated with repetitive strain injury.

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