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STRESS ENHANCES MUSCLE NOCICEPTOR ACTIVITY IN THE RAT

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Abstract—Chronic widespread pain, such as observed in irritable bowel (IBS) and fibromyalgia (FMS) syndrome, are markedly affected by stress. While such forms of stress-induced hyperalgesia are generally considered manifestations of "central sensitization," recent studies in patients with IBS and FMS suggest an additional, peripheral contribution. To examine the effect of stress on muscle nociceptor function, we evaluated activity in nociceptors innervating the gastrocnemius muscle in an animal model of chronic widespread pain, water avoidance stress, in the rat. This stressor, which produces mechanical hyperalgesia in skeletal muscle produced a significant decrease (~34%) in mechanical threshold of muscle nociceptors and a marked, ~two-fold increase in the number of action potentials produced by a prolonged (60 s) fixed intensity suprathreshold 10 g stimulus. Stress also induced an increase in conduction velocity from 1.25 m/s to 2.09 m/s, and increased variability in neuronal activity. Given that these changes, each of at least moderate magnitude, would be expected to enhance nociceptor activity, it is likely that, taken together, they contribute to the enhanced nociception observed in this model of stress-induced chronic widespread pain.

Key words: stress, skeletal muscle, hyperalgesia, peripheral neuropathy, nociceptors, conduction velocity.

Clinical conditions characterized by chronic widespread pain, such as irritable bowel syndrome (IBS), temporomandibular disorder (TMD) and fibromyalgia syndrome (FMS) are well recognized to be exacerbated by stressful life events (Delvaux, 1999; Giske et al., 2009; Korszun et al., 1998; Lembo et al., 1999; Lew et al., 2009; Martin et al., 2010; Paras et al., 2009; Wood, 2004). The mechanisms by which stress negatively impacts these patients have generally been considered to be at the level of the central nervous system, via changes in descending modulatory nociceptive controls (Porreca et al., 2002; Ren and Dubner, 2002; Vanegas and Schaible, 2004). The balance between descending inhibitory and facilitatory controls on spinal nociceptive circuits is believed to be affected by stress (Blackburn-Munro and Blackburn-Munro, 2001; Heinricher et al., 2009; Imbe et al., 2010; Martenson et al., 2009; Rivat et al., 2010; Yilmaz et al., 2010). The magnitude, duration and nature of the stress (e.g. continuous or intermittent/unpredictable or not) are determining factors, with recent evidence indicating that chronic stress facilitates pain transmission in animal models (Imbe et al., 2004, 2010) and in patients with chronic pain (Karp et al., 2008).

Recent experiments in patients with IBS and FMS have provided evidence for a further contribution by peripheral mechanisms. For example, application of local anesthetics, in the gastrointestinal tract in patients with IBS (Price et al., 2009; Verne et al., 2003) or to somatic tissues in patients with FMS (Staud et al., 2009), produces a widespread improvement in symptoms. Furthermore, in rodent models of IBS, a contribution of a peripheral mechanism has been suggested based on observations that chronic visceral hypersensitivity in adult rats, produced by colon irritation in neonates, is correlated with greater spontaneous firing, enhanced response to mechanical stimulation and lowered mechanical threshold in visceral afferents (Lin and Al-Chaer, 2003) and that intracolonic lidocaine decreases somatic as well as visceral nociceptive response in the trinitrobenzene sulfonic acid (TNBS) model of IBS (Zhou et al., 2008). To provide more direct evidence that sensitization of nociceptive afferents is a component of chronic widespread musculoskeletal pain syndromes we evaluated muscle nociceptor function in a model of stress-induced muscle hyperalgesia in the rat. Given the marked co-morbidity between IBS and FMS, we elected to use water-avoidance stress, which has been shown to produce visceral hyperalgesia (Bradesi et al., 2005, 2009; Hong et al., 2009; Yu et al., 2010), and more recently to produce mechanical hyperalgesia in the gastrocnemius and masseter muscles, as well as an anxiety phenotype on the elevated plus maze (Green et al., 2011).

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague-Dawley rats (250–350 g, Charles River, Hollister, CA, USA) were used in these experiments. They were housed in the Animal Care Facility at the University of California San Francisco, under environmentally controlled conditions (lights on 7:00–19:00 h, room temperature 21–23 °C) with food and water available *ad libitum*. Animal care and use conformed to NIH guidelines and experimental protocols were approved by the University of California San Francisco Committee on Animal Research.

Water avoidance stress

We used the water-avoidance model for irritable bowel syndrome (Bradesi et al., 2005), which we have recently shown, also pro-

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Abbreviations: CV2, coefficient of variation; FMS, fibromyalgia syndrome; IBS, irritable bowel syndrome; ISI, inter-stimulus interval.

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duces mechanical hyperalgesia in skeletal muscle (Green et al., 2011). Rats were placed on a 10 cm high acrylic platform (8×8 cm^2) in the center of a clear plastic tank (45 cm length×25 cm width×25 cm height) filled with room temperature tap water to a depth of 9 cm, for 1 h/d, for 10 consecutive days. The water-avoidance protocol produces a psychological stress as indicated by the large increase in adrenocorticotropic hormone and glucocorticoids within 30 min of the start of stress exposure (Bradesi et al., 2005). Single fiber electrophysiology was performed 1 day after the last stress exposure.

Single fiber recording

The *in vivo* single fiber electrophysiology technique employed was similar to that used previously in recordings from cutaneous afferents (Chen, et al., 1999). Rats were anesthetized with sodium pentobarbital (initially 50 mg/kg, i.p., with additional doses given throughout the experiment to maintain areflexia), their trachea cannulated, and heart rate monitored. Anesthetized animals were positioned on their right side and an incision made on the dorsal skin of the left leg, between the mid-thigh and calf, and the biceps femoris muscle partially removed to expose the sciatic nerve and gastrocnemius muscle. The edges of the incised skin were fixed to a metal loop to provide a pool that was filled with warm mineral oil, bathing the sciatic nerve and gastrocnemius muscle.

The sciatic nerve was cut proximally to prevent flexor reflexes during electrical stimulation of sensory neurons. Fine fascicles of axons were then dissected from the distal stump, and placed on a recording electrode. Single units were first detected by mechanical stimulation of the gastrocnemius muscle with a small blunt-tipped glass bar. Bipolar stimulating electrodes were then placed and held on the center of the receptive field of the muscle afferent, by a micromanipulator (MM-3, Narishige, Tokyo, Japan). Conduction velocity of each fiber was calculated by dividing the distance between the stimulating and recording electrodes by the latency of the electrically evoked action potential. All recorded muscle afferents had conduction velocities in the range of type III (12%) or type IV (88%) fibers. Mechanical threshold was determined with calibrated von Frey hairs (VFH Ainsworth, London, UK). The #10 (1.66 g) VFH was used first to elicit spikes, and if a response was elicited, then the #8 (0.603 g) was used. If this also elicited spikes, then the #6 (0.219 g) was used; if there was no response to #6, then #7 (0.363 g) VFH was used. Threshold is defined as the lowest force that elicited at least two spikes within 1 s, in at least 50% of trials. Sustained (60 s) suprathreshold (10 g) mechanical stimulation was accomplished by use of a mechanical stimulator that consisted of a force-measuring transducer (Entran, Fairfield, NJ, USA) with a blunt plastic tip that was applied by a micromanipulator (BC-3 and BE-8, Narishige) on the center of the receptive field, for 60 s. Neural activity and timing of stimulus onset and termination were monitored and stored on a Windows OS computer with Micro 1401 interface (CED, Cambridge, UK) and analyzed off-line with Spike2 software (CED).

Interspike interval (ISI) analysis

ISI analysis, used to evaluate the temporal characteristics of the response of C-fiber nociceptors to sustained mechanical stimulation, was adopted from our study of nociceptor activity in the rat model of vincristine-induced painful neuropathy (Tanner et al., 2003). The ISIs for the responses of each C-fiber were grouped into 100 ms bins between 0 and 499 ms; the few ISIs greater than or equal to 500 ms were not analyzed (Tanner et al., 2003). This bin width also allows comparison of data with that from previous studies (Arendt-Nielsen et al., 2000; Franck et al., 1993; Miller and Woolf, 1996). The number of intervals occurring in each bin were expressed as the percentage of the total number of ISIs in the trial. This normalization procedure allowed the distribution of ISIs from several fibers to be averaged together.

Coefficient of variation analysis

Coefficient of variation of the ISIs does not give an accurate estimate of the variability of neuronal firing if the mean rate changes over time, a common occurrence. Therefore, we calculated the coefficient of variability (CV2) which compares the relative difference between adjacent ISIs (Holt et al., 1996). CV2 is defined as the square root of two multiplied by the S.D. of two ISIs divided by their mean (Holt et al., 1996):

$CV2 = \frac{2|\Delta t_{i+1} - \Delta t_i|}{\Delta t_{i+1} + \Delta t_i}$, where *ti* is the latency for the i^{th} action potential.

Thus, CV2 is a dimensionless number that is independent of absolute firing rate.

Statistical analyses

Group data are expressed as mean±SEM of *n* distinct observations. Statistical comparisons were made by a Student's *t*-test (for one or two independent populations) or by one-way ANOVA for comparing multiple treatments, using Prism statistical software. To take uneven variances into account, for comparisons between groups of unequal numbers, Welch's correction for the Student's *t*-test was used. To compare CV2 analyses, a one-way repeated-measures ANOVA was used; $P<0.05$ was considered statistically significant.

RESULTS

Mechanical threshold

When tested by application of von Frey hairs to the peripheral receptive field in the gastrocnemius muscle, the mechanical threshold of muscle afferents in rats exposed to water avoidance stress (0.81±0.11 g, $n=26$) was significantly lower than the mechanical threshold of muscle afferents in naïve control animals (1.11±0.11 g, $n=40$, $P<0.05$, Student's *t*-test; Fig. 1). Thus, water-avoidance stress produces ~34% decrease in mechanical threshold for activation in skeletal muscle nociceptors.

Response to sustained stimulation

To examine excitability in muscle nociceptors from stressed rats, we evaluated their response to a sustained suprathreshold (10 g) von Frey stimulus. The response of muscle afferents to this sustained mechanical stimulation, in rats previously exposed to water avoidance stress (193.6±27.0 action potentials/60 s stimulus, $n=26$), was significantly greater than in afferents from control animals (116.8±15.7 action potentials/60 s stimulus, $n=40$, $P=0.005$, Student's *t*-test, Fig. 2); single-unit C-fiber recordings of action potentials evoked by a 10-g stimulus in naïve control and in water-avoidance stressed rats is shown in Fig. 2C. In rats exposed to stress, there was also an increase in firing frequency of muscle nociceptors for the first 8 s of the sustained 10 g stimulation (two-way repeated measures ANOVA, with Bonferroni post-hoc test, $P<0.05$, control vs. stress exposed, Fig. 3). Thus, water-avoidance stress produces a marked increase in the response of muscle nociceptors to mechanical stimulation.

Nociceptor firing pattern

To examine the pattern of neural activity in nociceptors from stressed animals we generated inter-stimulus interval