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, ~2-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.

Keywords

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[13,19,32,35,36,41,51,71]. 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 [52,57,67]. The balance between descending inhibitory and facilitatory controls on spinal nociceptive circuits is believed to be affected by stress [5,21,25,40,58,72]. 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 [24,25] and in patients with chronic pain [27].

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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 [54,68] or to somatic tissues in patients with FMS [62], 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 [38] and that intracolonic lidocaine decreases somatic as well as visceral nociceptive response in the trinitrobenzene sulfonic acid (TNBS) model of IBS [74]. 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 [6,7,23,73], and more recently to produce mechanical hyperalgesia in the gastrocnemius and masseter muscles, as well as an anxiety phenotype on the elevated plus maze [20].

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 07:00–19:00 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 [6], which we have recently shown also produces mechanical hyperalgesia in skeletal muscle [20]. Rats were placed on a 10 cm high acrylic platform (8 × 8 cm) 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/day, for 10 consecutive days. The water-avoidance protocol produces a psychological stress as indicated by the large increase in ACTH and glucocorticoids within 30 min of the start of stress exposure [6]. 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 [11]. 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, 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 (1.66g) 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 2 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 [63]. 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 [63]. This bin width also allows comparison of data with that from previous studies [2,18,46]. The number of intervals occurring in each bin was 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 [22]. CV2 is defined as the square root of 2 multiplied by the S.D. of two ISIs divided by their mean [22]:

Error! Bookmark not defined. $CV2 = \frac{2|\Delta t_{i+1} - \Delta t_i|}{\Delta t_{i+1} + \Delta t_i}$, where t_i is the latency for the ith 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 sec stimulus, n= 26), was significantly greater than in afferents from control animals (116.8 ± 15.7 action potentials/60 sec 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 (ISI) histograms and performed coefficient of variation (CV2) analyses for muscle afferents recorded in stressed and control rats. We found no difference in the ISI histogram for muscle afferents from stressed and control rats (Fig. 4). However, we did find a significantly greater CV2 (indicating increased interspike interval variability) for muscle afferents from rats exposed to water-avoidance stress ($P < 0.05$, two-way repeated measures ANOVA, Fig. 6). Thus, in addition to a significant decrease in mechanical nociceptive threshold and increase in response to sustained suprathreshold stimulation in muscle nociceptors from rats exposed to water-avoidance stress, there was also a difference in the firing patterns in these nociceptors.

Conduction velocity

The conduction velocity of muscle afferents in rats exposed to water avoidance stress (2.09 ± 0.30 m/sec, n= 26) was markedly faster than that of afferents in control animals (1.25 ± 0.11 m/sec, n= 40 $P < 0.05$, unpaired Student's t test with Welch's correction Fig. 5). When the distribution of conduction velocities in fibers of the two groups of animals (stress and control) was examined, a shift in the distribution of conduction velocities was observed. Thus, while not excluding a variation in percentage of a fiber type that was sampled, our data is most compatible with the suggestion that our findings are not explained by a larger percentage of type III afferents recorded in stressed rats (Fig. 5) (conduction velocities of type III and type IV fibers are 2.5 – 30 and <2.5 m/s, respectively [44]).

DISCUSSION

Clinical conditions characterized by widespread pain (e.g., IBS, TMD and FMS) are amongst the most common forms of chronic pain [12,53,64], as well as amongst the most difficult to treat [34,42]. The ability of stress to enhance pain has been well documented in a

large number of chronic pain syndromes [32,35,36,51] and in animal models of these conditions [28,29,33,49,70]. Indication of an involvement of CNS mechanisms in these pain syndromes arose, in part, from the observations of the major impact stress has on these clinical conditions [13,19,32,35,36,41,51,71], and a contribution of CNS mechanisms is further supported from experimental studies [43,61,69]. More recently peripheral nociceptive effects of stress axis mediators (catecholamines and glucocorticoids) [30] have raised interest in a peripheral contribution, as well. Since these latter studies of peripheral mechanisms have used behavioral or reflex measures of nociception, how these peripheral mechanisms are reflected in activity of nociceptive afferents has not been directly studied. Of note, in a rat model of chronic visceral hypersensitivity, there is greater spontaneous activity, response to mechanical stimulation and lowered mechanical threshold in visceral primary afferent fibers [38], as well as a contribution of central sensitization [1], suggesting a contribution of peripheral as well as central mechanisms. In the present experiments we examined skeletal muscle nociceptor function in an animal model of chronic widespread pain, water-avoidance stress, which produces both musculoskeletal [20] and visceral [37] hyperalgesia in the rat.

The psychological stress produced by water-avoidance, which induces robust mechanical hyperalgesia in skeletal muscle, also induced multiple changes in the function of muscle nociceptors, including ~34% decrease in mechanical threshold, an almost doubling of the number of action potentials produced by a sustained suprathreshold stimulus, an increase in interspike interval variability and an increase in nociceptor conduction velocity. Given that most if not all of these changes would, alone, be expected to be associated with enhanced nociceptor activity, taken together, we suggest that these changes in skeletal muscle primary afferent nociceptor function produced by chronic stress make a substantial contribution to musculoskeletal mechanical hyperalgesia in the water-avoidance stressed rat. Of note, alterations in cutaneous mechanical thresholds are not always observed and as such, these changes in muscle mechanical threshold may well occur more commonly for muscle nociceptor afferents

In the setting of inflammatory pain, neuronal conduction velocity has generally been found to be unchanged [4,47] and for peripheral neuropathies, even those associated with pain, most commonly an unchanged or slowing conduction velocity has been observed [17,45,47]. This paradox between expected and observed changes may be explained, at least in part, by the fact that in patients with peripheral neuropathies [48,60,65], and in animal models of neuropathic pain [3,8,9,26,45] conduction velocity is generally measured in myelinated, non-nociceptive, afferents. However, there are studies in which slowing conduction velocity of cutaneous C-fiber were observed in neuropathic models (e.g. dideoxycytidine [10]) and clinical studies with neuropathic pain (e.g. erythromelalgia [50]). Methodological differences may also play a role, due to differences in properties of cutaneous versus muscle C-fibers (e.g. cutaneous C-fiber conduction velocity slows markedly with repetitive stimulation [55,59]). Of note, in this regard, we have recently observed, in a model of cancer chemotherapy-induced painful peripheral neuropathy, produced by paclitaxel (Taxol; [14]), that while there was no change in conduction velocity in C-fibers innervating the hind paw there was an increase in conduction velocity of the same magnitude produced in the present study, in nociceptors innervating the gastrocnemius muscle [20]. While the underlying mechanism(s) responsible for the increased conduction velocity in muscle afferents is unknown, such changes must be due to changes in an ionic conductance in the axon. Changes in channel kinetics might also explain the significantly higher CV2 in muscle afferents from stressed rats. Djhouri and Lawson observed that chronic inflammation produced by intradermal injection of complete Freunds' Adjuvant increased C-fiber conduction velocity[16], suggesting that direct exposure of the peripheral nerve to the effect of cytokines might have produced an enhancement of conduction velocity in nociceptors. Possibly related to this, Gold and colleagues have recently shown that persistent

inflammation alters the density and distribution of voltage-activated ion channels in DRG neurons [39].

The mechanism by which stress produces such marked changes in nociceptor function are, at the moment, unknown. However, chronic stress is known to upregulate Na^+ channels [66], and the sympathoadrenal stress hormone, epinephrine, enhances tetrodotoxin-resistant Na^+ currents in dorsal root ganglion neurons [31]. We have provided evidence that stress can induce neuroplasticity in peripheral sensitization mechanisms in skin and muscle in the rat [28-30,56] and even induce mechanical hyperalgesia [20]. These effects of stress are due, at least in part, to neuroendocrine stress axis mediators, catecholamines and corticosteroids [29], acting on adrenergic and glucocorticoid receptors on sensory neurons [28]. And, in a model of alcohol-induced painful peripheral neuropathy, we have shown that alcohol consumption fails to produce mechanical hyperalgesia in adrenal medullectomized rats [15].

CONCLUSION

This is the first demonstration of stress-induced changes in primary afferent nociceptor function in skeletal muscle, produced by analysis of nociceptor activity in a model of stress-induced musculoskeletal hyperalgesia in the rat. Stress produced a modest decrease in mechanical nociceptive threshold, an increase in the number of action potentials produced in response to a prolonged stimulus, and an increase in conduction velocity of muscle nociceptors. Given that these changes in nociceptor function would be expected to predispose to increased activity in muscle nociceptors and are at least moderate in magnitude, it is likely that they make a substantial contribution to the enhanced nociception observed in this model of chronic widespread pain.

Acknowledgments

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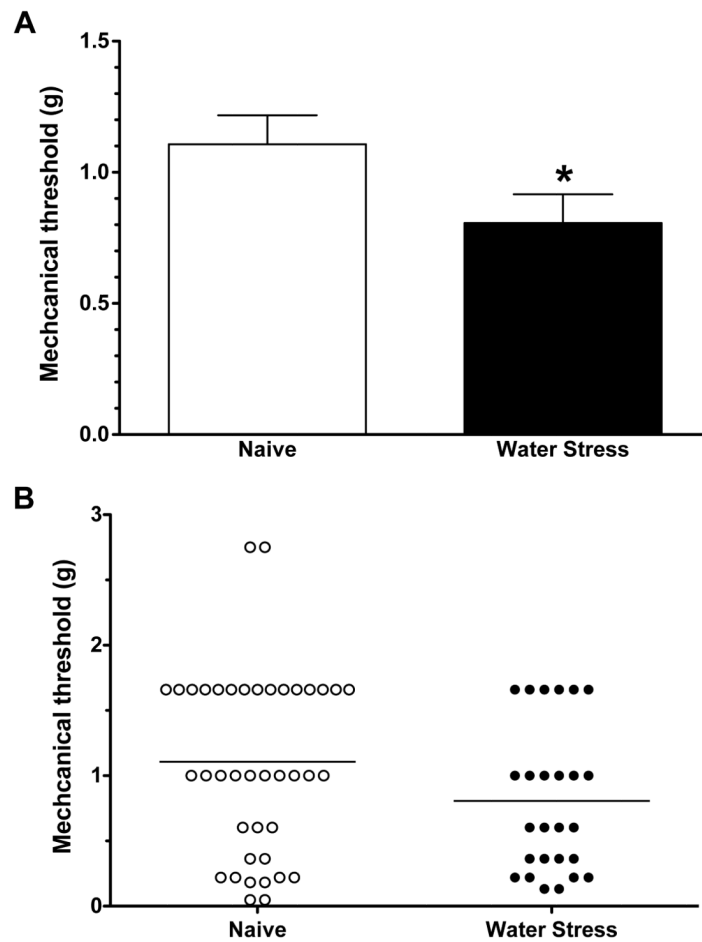
Figure 1.
A. Mechanical threshold of nociceptors innervating the gastrocnemius muscle, from water-avoidance stress-exposed (n = 26) and control rats (n = 40). Muscle nociceptors in water-avoidance-exposed rats had significantly lower mechanical thresholds than nociceptors from naïve control rats (P<0.05, Welch's correction for the Student's t-test).
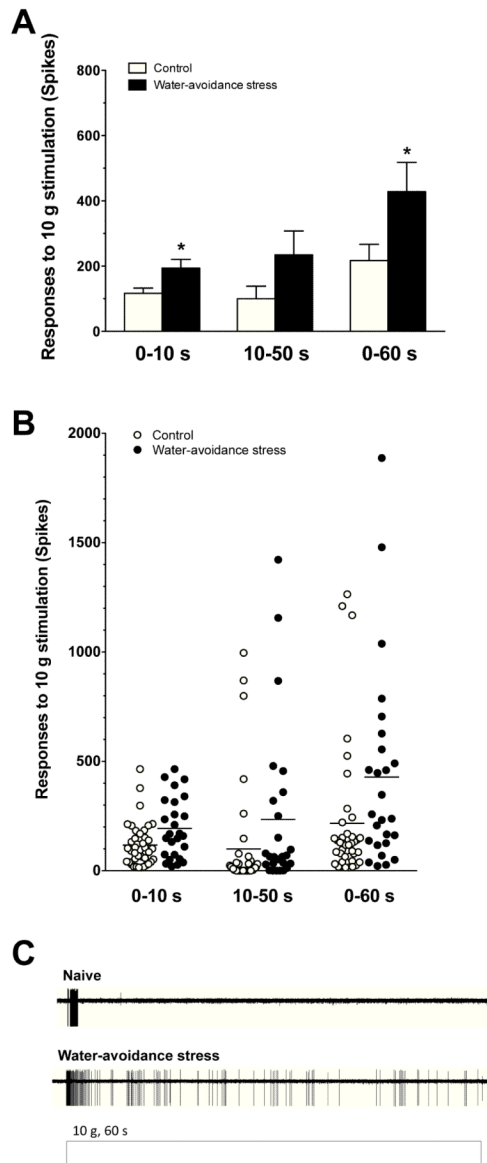**B.** Scattergram of mechanical thresholds of individual muscle nociceptors from naïve control and water-avoidance stress-exposed rats.

Figure 2.
The response of nociceptors to sustained (60 s) suprathreshold (10 g) von Frey hair mechanical stimuli in muscle nociceptors. Mean values (**A**) and individual fibers (**B**) are shown from naïve control and water-avoidance stress-exposed rats. The responses of the nociceptors from the water-avoidance group ($n = 26$) were significantly higher (for 0-10s and 0-60s) than those of control rats ($n = 40$, *$P<0.05$, Welch's correction for the Student's t-test). (**C**) Single-unit C-fiber recordings of action potentials evoked by a 10-g stimulus in naïve control and in water-avoidance stressed rats.
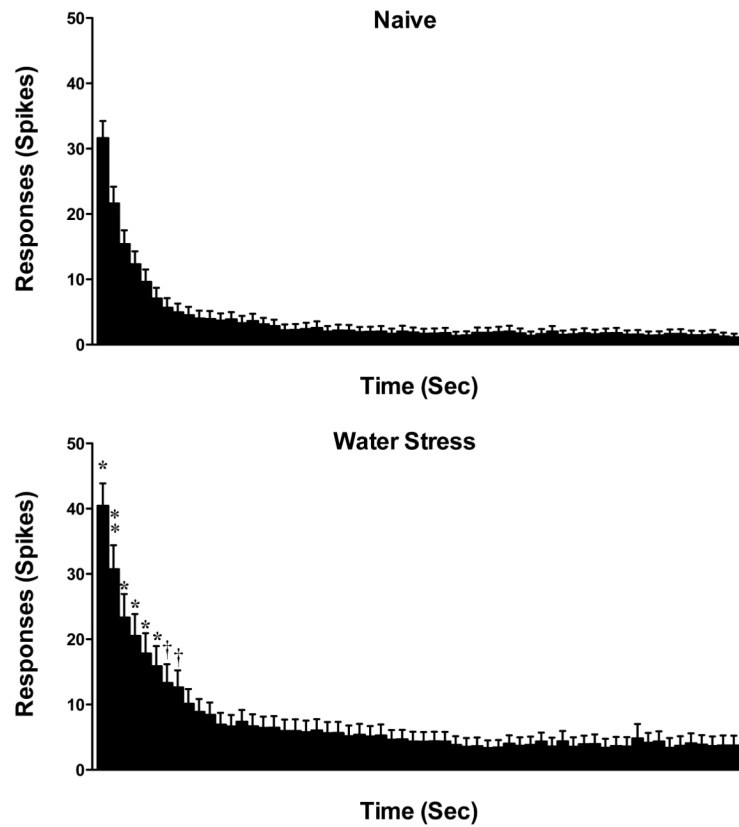
Figure 3.
The time course of the average responses of nociceptors during the 60 s suprathreshold stimulus, for naïve control (n = 40) and water-avoidance stressed (n = 26) rats; bin width 1 s. The number of action potentials in muscle afferents from stressed rats was significantly greater during the first $1 - 8$ s stimulus period (two-way repeated measures ANOVA, with Bonferroni post hoc test; † $P<0.05$; * $P < 0.01$; and, □ $P<0.001$; naïve control vs. stress).
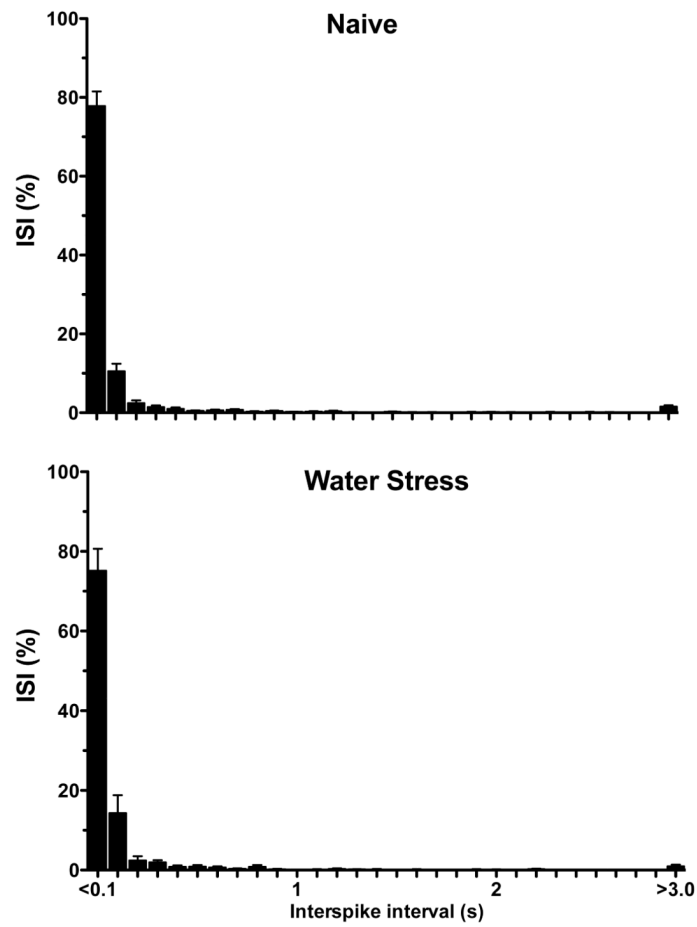
Figure 4.
The inter-stimulus interval (ISI) distribution of muscle nociceptors in response to sustained (60 s) suprathreshold (10 g) von Frey hair mechanical stimuli, for water-avoidance stress-exposed (n = 26) and naïve control (n = 40) rats (bin width 1 s).

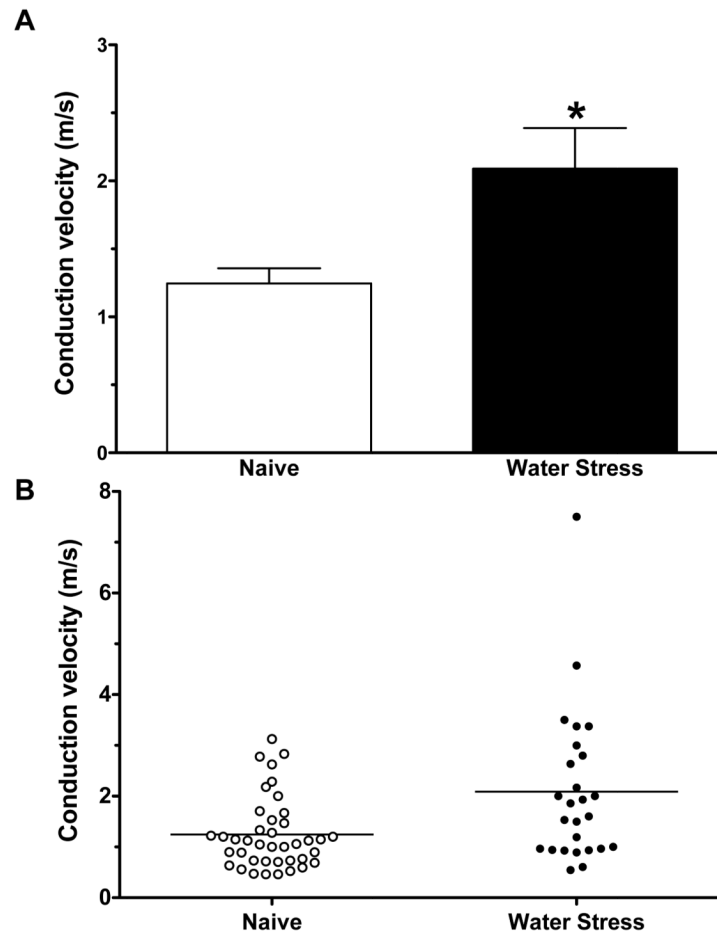**Figure 5.**
A. Mean conduction velocities from water-stressed rats (2.09 ± 0.3 n=26) were significantly greater than from naïve rats (1.25 ± 0.11 n=40, Welch's correction for the Student's t-test, p < 0.05).
B. Scattergram of conduction velocities of muscle nociceptors in naïve control and water-avoidance-treated rats.
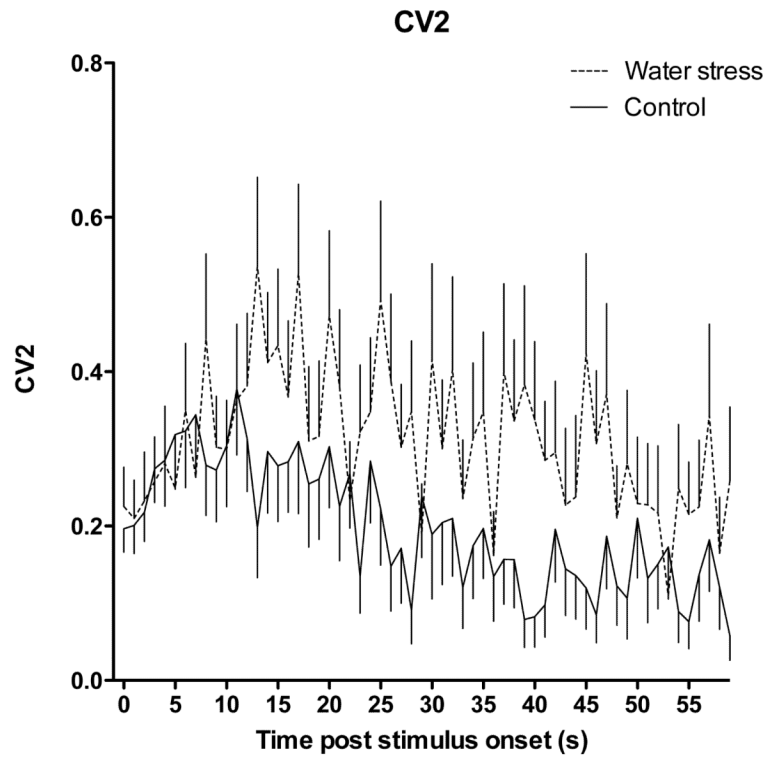
Figure 6.
The coefficient of variation (CV2) distribution of muscle nociceptors from water-avoidance stress-exposed (n = 26) and naïve control (n = 40) rats, in response to sustained (60 s) suprathreshold (10 g) von Frey hair mechanical stimulation. CV2 is significantly greater in neurons of rats exposed to water-avoidance stress, compared to naïve controls (two-way repeated measures ANOVA, P<0.05).