Modulation of Musculoskeletal Hyperalgesia by Brown Adipose Tissue Activity in Mice

A thesis SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA BY:

Elizabeth M. Goudie-DeAngelis, DVM

IN PARTIAL FUFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

Alice Larson, PhD

August 2016

Acknowledgements

Ramy E. Abdelhamid, Myra G. Nunez, Casey L. Kissel, Katalin J. Kovács, Robert Larson, Philip S. Portoghese, and Alice A. Larson

Initial experiments evaluating mice injected with Beta-agonist (BRL37344) were performed by Ramy E. Abdelhamid. All other experiments were performed by the author and Casey L. Kissel with scribe work performed by Robert Larson, and data analysis assistance by Myra G. Nunez.

This work was supported by a grant from NIH from the National Institutes on Arthritis and Musculoskeletal and Skin Diseases [AR056092] and support from the College of Pharmacy and College of Veterinary Medicine at the University of Minnesota.

Abstract

Brown adipose tissue plays an important role in thermogenesis and metabolism in response to stress. Acutely, stress induces thermogenesis by increasing sympathetic tone to beta$_3$ (β_3) adrenergic receptors in brown adipose. Chronic stress leads to the hypertrophy of brown adipose, a phenomenon termed adaptive thermogenesis. Cold exposure and the associated sympathetic activity from a variety of stresses are also known to increase pain in patients suffering from painful disorders such as fibromyalgia syndrome. We theorized an association between brown adipose tissue activity and musculoskeletal hyperalgesia and tested this hypothesis in mice. Exposure to a cold swim stress enhanced musculoskeletal hyperalgesia, as indicated by morphine-sensitive decreases in grip force responses. Stimulation of β_3 adrenergic receptors by injection of BRL37344, a β_3 adrenergic agonist, also enhanced musculoskeletal hyperalgesia, consistent with the activation of the unique set of adrenergic receptors located in brown adipose. Chemical ablation of interscapular brown adipose, using Rose Bengal, attenuated the development of hyperalgesia in response to either swim stress or BRL37344. Similarly, elimination of the gene expressing uncoupling protein-1 (UCP1), the enzyme responsible for thermogenesis, prevented musculoskeletal hyperalgesia in response to a swim stress, as documented in UCP1-knock out (UCP1-KO) mice compared to wild type controls. Together these data provide a convergence of several lines of evidence suggesting that the acute activation of brown adipose contributes to muscloskeletal hyperalgesia. However, the baseline nociceptive sensitivity of UCP1-KO mice was greater than wild type controls, suggesting

that a mechanism that promotes muscle pain is present that compensates for the absence of UCP1.

Table of Contents

List of Figures

1. Introduction

Brown adipose tissue, white adipose tissue, and beige adipose tissue are three types of fat found in the mammalian body. Until about 20 years ago, it was assumed that brown adipose tissue had little to no importance in adult mammals despite its wide distribution throughout the body. Although descriptions of this tissue can be traced back to as early as 1551,[6] the study of its function and recognition of this organ's significance is relatively new. Recent studies emphasize the physiologic importance of brown adipose tissue. [6] The majority of beta-3 (β_3) adrenergic receptors is located in brown adipose tissue as well as gall bladder, urinary bladder, brain, but they are absent in skeletal muscle.[8; 20] Both thermogenesis and metabolism rely heavily on the sympathetic stimulation of β_3 adrenergic receptors in brown adipose tissue. [15; 21; 35] In adult animals, exposure to acute cold-stress can stimulate thermogenesis through brown adipose tissue activity. Non-shivering thermogenesis is mediated by uncoupling protein-1 (UCP1), or thermogenin, which is highly concentrated in the mitochondria of brown adipocytes. [6; 14; 25; 37] After chronic exposure to cold or other stress, UCP1 activity increases the mass of brown adipose tissue by upregulating the synthesis of UCP1. [22; 27]

Musculoskeletal hyperalgesia is observed in patients with fibromyalgia syndrome, a chronic pain disorder that is not responsive to anti-inflammatory medications. Women are more frequently afflicted with fibromyalgia than men. The disease is not only characterized by widespread pain, but also a lower than normal body temperature, [6] and a decreased adrenal gland response of the hypothalamic-pituitary-adrenal (HPA) axis to stress [10] and enhanced activation of the sympathetic nervous system to stress [36]. Additionally, fibromyalgia sufferers exhibit a hyperalgesic response to norepinephrine.[24] The tactile and musculoskeletal pain experienced by patients with fibromyalgia is worsened by exposure to even mild daily stress and exposure to cold. Transient relief of fibromyalgic musculoskeletal pain is provided by heat. [23] In addition to analgesic drugs, treatment of the disease includes strategies to decrease daily stress and

gradually increasing physical exercise. The sensitivity to stress and analgesic effect of heat suggests a possible link between the musculoskeletal hyperalgesia in patients with fibromyalgia and alterations in brown adipose tissue activity.

Positive associations between stress, sympathetic stimulation, increased nociception, and thermoregulatory changes after exposure to extreme cold or stress even exist in healthy individuals. [16] Musculoskeletal hyperalgesia can be induced in animal models in response to acute stressors, including a forced swim in rodents.[1; 28; 34; 38] Acute stress leads to increased sympathetic tone with an associated release of catecholamines that stimulate adrenergic receptors.[11; 30] Furthermore, increased sympathetic tone propagates the complex regional pain syndrome in which non-traumatized parts of the body experience allodynia and hyperalgesia.[4] In addition to allodynia and hyperalgesia, psychological stress-induced hyperthermia can be induced via catecholamine activation of β_3 adrenergic receptors in brown adipose tissue.[15] An increase in brown adipose tissue mass and activity is also induced by chronic stress that does not involve cold-exposure. It is known that nociceptive afferent fiber and temperature-sensitive afferents share common neurotransmitters, such as substance P, [12] in collateral populations of brown adipose tissue found throughout skeletal muscle [2], and that temperature and nociception share common regulatory pathways in the central nervous system . Whether stress-induced increases in brown adipose activity correspond temporally and are located in anatomically similar areas and regulated by similar neurotransmitters as those that influence nociception, is not known. Likewise, it is unknown if brown adipose activity influences pain. We posited a link between the activation of the brown adipose tissue and musculoskeletal hyperalgesia based on the sensitivity of this modality of pain to stress-induced hyperalgesia**.** The goal of this study was to determine whether brown adipose tissue activity contributes to the development of stress-induced mechanical hyperalgesia using a model that we have previously characterized in mice.[1]

We hypothesized that stress-induced activation of brown adipose tissue contributes to the

development musculoskeletal hyperalgesia. To test this hypothesis, we examined whether ablation of interscapular brown adipose tissue, a major depot of brown adipose in rodents, alters the generation of musculoskeletal hyperalgesia normally observed after stress.[1] Among its many effects, sympathetic activity mediates stress-induced activation of brown adipose.[35] There, release of catecholamines activate β3 adrenergic receptors, a subtype that is located in only a handful of locations in the body.[8] Based on this we further postulated that sympathetic activity modulates the musculoskeletal hyperalgesic effect of swim stress. To test this, we examined the ability of BRL37344, a drug that selectively activates β_3 adrenergic receptors, that are densely located in brown adipose, to mimic the hyperalgesic effect of stress and the tendency for ablation of brown adipose to prevent this effect. Because UCP1 is the primary enzyme responsible for thermogenesis, we then compared the ability of stress to induce musculoskeletal hyperalgesia in UCP1-KO mice to that in wild type control mice. Together our data suggest that activation of brown adipose tissue contributes to musculoskeletal hyperalgesia in mice.

2. Materials and Methods

2.1 Animals

Adult female and male Swiss Webster mice (Harland Sprague Dawley INC, Indianapolis, MN) weighed 20-25 g. UCP-1 knock out mice and their wild type littermate control C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were all female weighing 13-15 g. All mice were housed 3 to 5 mice per cage and allowed to acclimate for at least one week prior to use. Free access to water and food was allowed during acclimation and the room was maintained at a constant temperature of 23^{o}C on a 12-h light-dark cycle. The estrus cycle of the female mice was not taken into account when comparing results. All procedures were performed according the to the guidelines of the International Association for the Study of Pain (IASP), the University of Minnesota Animal Care and Use Committee, and the Committee of Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication NIH 78-23, revised 1995) and approved by the University of Minnesota Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternative in vivo techniques, when possible.

2.2 Drugs and chemicals

(R*, R*)-[4-[2-[[2-(3-Chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid sodium hydrate (BRL37344), a β_3 adrenergic agonist, was obtained from Tocris Bioscience (Minneapolis, MN), dissolved in Nanopure water (pH 4.0-4.5) and administered subcutaneously (s.c.) at a dose of 1 mg/kg and later doses of 3 mg/kg and 10 mg/kg in the UCP1 knock-out mice experiments. [29] The primary metabolite of BRL37344 induces increased energy expenditure in brown adipose tissue of mice.[7] Morphine sulfate, a μ-type opioid receptor agonist, was purchased from Mallinckrodt (St. Louis, MO). Morphine was diluted in saline (pH 5.0) and injected intraperitoneally (i.p.) at a dose of 10 mg/kg that has strong antinociceptive effects in preliminary studies employing the thermal tail flick assay in water maintained at either 49^{o}C or 53^{o}C.

2.3 Partial ablation of brown adipose tissue

Rose Bengal dye was obtained from Sigma-Aldrich (St. Louis, MO) and injected at a dose of 10 mg/kg into the vascular system by an intracardiac stick while mice were anesthetized using isoflurane. The area of the interscapular brown adipose tissue was then exposed, isolated and exposed to an intense light source for 10 min using a Fiber Optic Illuminator from Cole-Parmer (Vernon Hills, IL). The high intensity lamp (150 watt quartz halogen lamp with fiber optic diameter of 0.3 mm and light intensity set at 50%) is reported to induce the local generation of Rose Bengal free radicals that cause a local degeneration of tissue resulting in a partial ablation where illuminated.[42] Sham mice were injected with dye and the brown adipose tissue isolated surgically, but not exposed to the high intensity illumination.

2.3 Forced swim

A forced swim was used as a form of acute stress as this has previously been found to influence mechanical nociception. [31-33; 38; 39] Each mouse was placed, individually, into a 2-L beaker (diameter: 15 cm, height: 20 cm) containing 18 cm of water that was maintained at 26^{o}C. This depth was sufficient that mice could not touch their feet or tail to the bottom. Grip-force was evaluated after a 15-min swim and at various time intervals thereafter to establish the time-course of the resulting hyperalgesia, as we have described previously.[1] Forced swim were repeated daily and the grip force values after each swim were routinely compared to other control groups or, where indicated, to their baseline measurements.

2.3 Grip-force assay

Forelimb grip force was measured using a force transducer as previously described in rats [18] and in mice.[17] The grip force apparatus consists of a force transducer connected to a wire mesh grid (12 x 7 cm^2 outer diameter with a 0.5 cm^2 wire grid) approximately 30 cm above the bench top. During testing, each mouse was held by the base of its tail and gently passed in a

horizontal direction over the wire grid until it grasped the grid with its forepaws. The force exerted by the forelimbs of each mouse when pulling on the grid was recorded by the force transducer. Grip force data are expressed in terms of grams (g). Two grip force measurements were obtained at each time point and the average of these measurements was used to represent each mouse's forelimb grip force at a particular time. Mice were familiarized with the grip force measurement procedure for three days prior to the experiment. On the third day, grip force measurements obtained prior to each intervention were used as baseline values for each animal.

2.3 Weighing of BAT

In a subset of mice, both experimental animals and controls were sacrificed after manipulations and their interscapular brown adipose depot removed and weighed.

2.3 *Tail Flick Assay*

While animals were manually restrained, the tail was submerged in a water bath maintained at a temperature of 49° to 51°C. The withdrawal latency was defined as the time for the animal to withdraw its tail from the water. To avoid tissue damage, a cut-off time of 15 sec was used.

3. Results

3.1 Relationship of brown adipose tissue to swim-induced hyperalgesia

To assess the influence of stress on musculoskeletal hyperalgesia, grip force responses were measured immediately after a 15-min forced swim at 26^{o}C. To differentiate between responses to thermogenesis (acute stress) and adaptive thermogenesis (chronic stress), the same mice were subjected to the forced swim and grip force measurements each day for 15 days. Grip force responses of mice subjected to the swim were lower than in the unstressed control group (Figure 1) when compared on the first day as well as on each day throughout the 15-day study (Figure 2A). Over this two-week period, decreases in grip force were maximal on day three and persisted until day 15.

Rectal temperatures of mice taken immediately following the grip force assay indicate decreased core body temperatures due to exposure to the swim (Figure 2B). The lowest value was on day one in response to the first forced swim, after which the ability to defend against the cold improved during the remaining daily swims.

Increases in the mass of brown adipose have been associated with increased thermogenic activity brought about by the process of adaptive thermogenesis.[15] To determine whether a daily swim for two weeks was sufficient to initiate this process, experimental and control groups were sacrificed on day 15, 24 h after the last swim, and their interscapular brown adipose extracted and weighed. This depot of tissue was heavier in mice exposed to a daily swim than in unstressed control mice (Figure 2C).

3.2 Effect of a β_3 adrenergic agonist on mechanical hyperalgesia

Based on the high density of β_3 adrenergic receptors in brown adipose and their absence in muscle, we postulated that β_3 adrenergic activity is sufficient to increase pain and that activation

of these sites would mimic the hyperalgesic effect of stress on musculoskeletal nociception. To determine the effect of β_3 adrenergic receptor activity on musculoskeletal nociceptive responses, BRL37344 was injected s.c. at a dose of 1 mg/kg, a dose that was sufficient to increase rectal temperatures 15 min after its injection (Figure 3C). Grip force responses were measured before as well as 60 min (Figure 3A) and 24 h (Figure 3B) after the first (Figure 1) and after each of the remaining 14 daily injections (Figure 3B). At 60 min after the first injection, BRL37344 signficantly decreased grip force responses compared to their baseline values (paired Student's t-test) while saline did not. However, there was no difference between the first three daily responses in the BRL37344 group and saline-injected controls (Figure 3A), suggesting that the stress of the injections was sufficient to influence grip force measures. When measured 24 h after each daily injection, grip force responses of the group injected with BRL37344 were still decreased on days 1-5 whereas vehicle-injected control mice were no longer affected (Figure 3B). After the first week of testing, habituation to this effect of BRL37344 developed as there was no longer a difference between grip force values of BRL37344-injected mice compared to vehicle-injected controls 24 h after their injection. No such tolerance developed to the ability of BRL37344 to increase rectal temperatures 15 min after its injection (Figure 3C).

To confirm that the dose of BRL37344 was sufficient to stimulate β_3 adrenergic receptors in brown adipose, we removed and weighed the interscapular brown adipose depot 24 h after the final injection of BRL37344 or vehicle. This depot was heavier in mice injected with BRL37344 than in vehicle-injected controls (Figure 3D), consistent with hypertrophy secondary to chronically increased tissue activation and adaptive thermogenesis.

3.3 Confirmation that decreases in grip force reflect hyperalgesia

To confirm that decreases in grip force values produced by an adrenergic agonist were due to mechanical hyperalgesia and not musculoskeletal weakness, groups exhibiting decreases in grip force were then injected with 1 mg/kg of BRL37344 for 3 days, 3 mg/kg for 3 days and 10 mg/kg for 2 days. After confirming that the BRL37344-injected mice were hyperalgesic at this time, as

reflected by a significant decrease in grip force responses (Figure 4), they were injected i.p. with either saline or 10 mg/kg of morphine and retested 30 min later, a morphine-challenge previously used to test swim stress-induced hyperalgesia. [1] The decrease in grip force 4 h after the last injection of BRL37344 was reversed by injection of morphine but not saline. Control mice that were not injected with BRL37344 were unaffected by morphine, consistent with the inability of morphine to enhance grip force responses above those of normal healthy mice. Together these data confirm that, like stress-induced hyperalgesia,[1] BRL37344-induced decreases in grip force responses reflect musculoskeletal hyperalgesia rather than weakness.

3.4 Effect of Rose Bengal ablation of interscapular brown adipose on hyperalgesia

To determine whether activation of brown adipose tissue is necessary for the increased mechanical hyperalgesia observed after a forced swim, the brown adipose of 12 mice was partially ablated using injection of Rose Bengal followed by illumination of the interscapular brown adipose depot, as described in methods.[42] Sham-operated controls were similarly injected but not illuminated. Two weeks later, when exposed to the first forced swim, mice whose interscapular brown adipose was ablated exhibited stronger grip force responses immediately after the swim than did sham-operated controls (Figure 1). When tested daily, these differences persisted for the first 5 days of the two-week study (Figure 5A).

To determine if it is the activation of β_3 adrenergic receptors that are located in brown adipose tissue that is necessary for the development of mechanical hyperalgesia in response to BRL37344 (as opposed to β_3 adrenergic receptors elsewhere in the body), interscapular brown adipose depots were similarly ablated in one group of mice while those in the other group were sham-operated and served as controls. All animals were then injected s.c. daily for 15 days with BRL37344 and grip force measured before as well as 60 min and 24 h later. Baseline grip force values were unaffected by prior ablation when compared with sham-ablated mice. However, mice with chemically ablated brown adipose depots exhibited no decrease in their mean grip force values 60 min after injection of BRL36344 while sham-operated mice were significantly

hyperalgesic when compared to their corresponding baseline control values (Figure 1) and 24 h (data not shown). These differences in response to BRL37344 persisted and were even greater throughout the remaining two weeks of daily injections and testing (Figure 5B).

Success of chemical ablation on intrascapular brown adipose tissue was confirmed by measuring the weight of this depot at the end of the study. Although daily swim and daily injection of mice with BRL37344 each increased the weight of interscapular brown adipose in the first studies (Figures 2 and 3), the interscapular brown adipose depot of mice whose tissue was chemically ablated using Rose Bengal weighed less than that of mice whose interscapular brown adipose was not ablated, whether the mice were exposed to daily swims (Figure 5C) or daily injection of BRL37344 (Figure 5D).

3.5. Comparison of body weight and temperature of UCP1-KO and wild type (WT) mice

To examine the necessity of uncoupling protein-1 (UCP1) activity in brown adipose tissue on the development of musculoskeletal hyperalgesia, we compared swim stress-induced hyperalgesia in UCP1-KO mice to those of UCP1-replete wild type (WT) controls. The average rectal temperature of UCP1-KO mice did not differ from WT controls when taken at the outset of the study (Figure 6B Baseline), consistent with the inactivity of brown adipose activity during thermoregulation at rest. However, temperatures of UCP-KO mice taken immediately after the first swim at 26°C were lower than WT controls, indicating a greater ability of mice with UCP1 to defend against the cold than those whose brown adipose function is deficient by the deletion of the UCP1 gene (Figure 6B after swim). UCP1-KO mice did not differ from WT controls in their body weight on the first day of the study (Figure 6A) and both WT and UCP1-KO mice became heavier, increasing their weight to a similar degree over the course of the 21-testing.

When evaluated immediately after a forced swim, grip force values of WT mice decreased significantly when compared to their pre-swim values on that day (Figure 1), in agreement with our results in Swiss Webster mice (Figure 1).[1] In contrast, grip force responses of UCP1-KO

mice to a forced swim were not significantly different than those of WT controls and not significantly different when compared to their own pre-swim baseline values. In a similar fashion, injection of UCP1-KO and WT mice with BRL37344 resulted in significantly different responses in the grip force assay between the groups.

Because UCP1 plays a role in thermoregulation during a challenge of cold or stress, we questioned whether nociceptive measures that are sensitive to body temperature, like the tail flick latency, might also be impacted by a deficiency of UCP1. In contrast to grip force values, tail flick latencies did not differ between UCP1-KO and WT mice.

4. Discussion

Based on overlapping patterns of innervation of thermal regulatory and nociceptive pathways as well as on the high sensitivities of both body temperature and of pain to stress, we theorized that a relationship may exist between brown adipose activity and stress-induced increases in the intensity of musculoskeletal pain.[15; 21; 24] The present study supports this hypothesis as musculoskeletal hyperalgesia produced by swim stress and replicated by β_3 adrenergic receptor activity, is attenuated by ablation of a large depot of brown adipose tissue and by the elimination of UCP1 gene expression in UCP1-KO mice. Together these findings suggest that the development of stress-induced musculoskeletal hyperalgesia is dependent, in part, on activation of brown adipose tissue or on pathways regulating the activity of this tissue.

Brown adipose can be found in many sites throughout the body, however, the interscapular depot is a major contributor to thermogensis in rodents.[6] To assess the importance of this system on muscle pain, we eliminated this depot using light-activated Rose Bengal, a model of tissue ablation that has been successfully used to destroy the olfactory bulb in mice.[42] Chemical ablation of the interscapular brown adipose depot had no effect on baseline grip force responses, confirming that in the absence of stress to initiate thermogenesis, brown adipose and its associated neuronal regulation is not involved in musculoskeletal nociceptive sensitivity and that ablation alone does not influence the ability of mice to grip. Ablation was successful based the absence of an increase in the weight of the interscapular brown adipose depot after two weeks of daily swims or of treatment with BRL37443 when compared to those in sham-operated controls. Ablation of this depot was sufficient to attenuate the hyperalgesia produced by a swim stress. In a similar fashion, molecular deletion of the UCP1 gene necessary for thermogenesis, as in UCP1-KO mice, prevented the decrease in grip force usually caused by a forced swim. Together these two lines of evidence, produced using different strategies to interfere with brown adipose activity,

converge to support the conclusion that swim stress-induced musculoskeletal hyperalgesia is dependent, in part, on intact brown adipose tissue.

We also found that injection of the β_3 adrenergic agonist BRL37443 mimicked the musculoskeletal hyperalgesic effect of swim stress. The decrease in grip force produced by BRL37443 was also reversed by morphine, similar to the decreases in grip force produced by swim stress.[1] This suggests that it is musculoskeletal hyperalgesia and not weakness that results from enhanced sympathetic activity as morphine is incapable of enhancing strength but has potent antinociceptive activity. Importantly, BRL37443-induced hyperalgesia was also attenuated by prior ablation of the interscapular brown adipose depot. This supports the possibility that it is adrenergic activation specifically in brown adipose that leads to musculoskeletal hyperalgesia. These studies specifically identify sympathetic activation of brown adipose as an important contributor to musculoskeletal hyperalgesia and perhaps to this model of stress-induced hyperalgesia.

Many types of stress are capable of activating brown adipose tissue.[3; 15; 21] For example, exposure of rats to cold for just two days causes brown adipose tissue to hypertrophy.[5] In mice, repeated cold exposure increases blood flow and UCP1 content in brown adipose, a change believed to reflect adaptive thermogenesis.[13] Although direct measurement of UCP1 or thermogenic activity would be necessary to prove an associated change in function, the increased weight of interscapular brown adipose that we observed after two weeks of daily swims suggests that the swim stress used in our protocol is sufficient to activate this tissue and even initiate adaptive thermogenesis. Similar increases after daily injections of BRL37443, a β_3 adrenergic receptor agonist, likely reflect activation of this tissue, similar to that produced by the elevated circulating catecholamines associated with Pheochomocytoma.[21]

Tolerance developed to musculoskeletal hyperalgesia induced by either a daily forced swim or by a daily injection of the β_3 adrenergic agonist. Tolerance may reflect the action of feedback

regulatory mechanisms that modulate the long-term influence of stress on muscle pain sensitivity. In contrast to chronic mild stress, a single intense stress (sham surgery) prolonged the time during which hyperalgesic responses to stress persisted. This may be due to a failure to develop tolerance to stress-enhanced muscle pain. Regardless of the mechanism, a sudden stress may contribute to the etiology of fibromyalgia pain as anecdotal reports suggest that prior trauma or episodes of intense stress precede the onset of this condition.

Elimination of UCP1 in KO mice had no effect on body weights and body temperatures, at rest, compared with those of wild type controls; consistent with previous studies.[14] Thermal nociception measured using the tail flick assay was unaffected by the absence of UCP1, suggesting that these mice do not have a heightened response to all types of pain

Our findings implicate brown adipose tissue in the generation of stress-induced musculoskeletal hyperalgesia in normal healthy mice. The exact mechanism(s) by which brown adipose increases musculoskeletal pain sensitivity is unclear, one possibility involves deferred pain carried along axon collaterals that project to brown adipose as well as surrounding skeletal muscle.[37] These nerves may be either sympathetic or primary afferent C-fibers, as both innervate muscle as well as brown adipose [40] and both are associated with hyperalgesic conditions. Stress activates both sympathomedullary and the hypothalamic-pituitary-adrenocortical axis. However, manipulation of glucocorticoids fails to alter musculoskeletal hyperalgesia in response to lipopolysaccharides, a chemical form of stress suggesting that the sympathetic axis of the stress reponse curve is not involved.[17] In contrast, sympathetic activity plays a vital role in the modulation of chronic pain states[26; 37] For example, rats exposed to sound stress develop mechanical hyperalgesia that is counteracted by prior elimination of sympathetic activity.[19] In contrast, increases in sympathetic tone result in worsening of clinical signs in chronic pain states like fibromyalgia and complex regional pain syndrome.[4; 9; 19; 24]

In addition to increased sympathetic activity causing hyperalgesia, primary afferent C-fibers may

also contribute specifically to musculoskeletal hyperalgesia. Desensitization of primary afferent fibers would be predicted to increase brown adipose tissue activity by interfering with its feedback inhibition.[41] While desensitization leads to a widely described protracted thermal antinociception desensitization of TRPV1 sites using resiniferatoxin, that is sufficient to cause thermal antinociception for 60 days, but also causes musculoskeletal hyperalgesia in the same mice for 30 days.[1] This cannot be explained well based on the innervation of these fibers to muscle as they are not dense in this tissue. However, these studies are consistent with the possibility that enhanced brown adipose tissue activity contributes to enhanced muscle pain either by enhanced sympathetic tone or by an inhibition of primary C-fiber activity, either of which would enhance thermogenesis. At this time further studies evaluating the specific mechanism involved are needed.

5. Conclusions

Our data confirm that musculoskeletal hyperalgesia, as measured using the grip force assay in mice, can be transiently induced by stress or by β_3 adrenergic receptor activity. Because this type of hyperalgesia is attenuated by prior ablation of brown adipose tissue or by elimination of UCP1 enzymatic activity in mice, brown adipose activity may be similarly linked to stress-induced increases in musculoskeletal pain in humans. While brown adipose is typically associated with thermogenesis, and chronic swim stress leads to adaptive thermogenesis, brown adipose appears to support the hyperalgesic effect of acute stress in a fashion that is not dependent on body temperature per se.

Figure Legends

Figure 1

Summary of the effect of swim stress (Swim) or injection of BRL37344 (BRL), a β3 adrenergic receptor agonist, on the average change in grip force responses (±SEM) compared to mice that did not swim (C) or were injected with vehicle (Veh). Mice were also forced to swim (panel A) or injected with BRL (panel B) following ablation of interscapular brown adipose tissue (Abl) compared to mice that were sham-operated (Sham). All mice were Swiss Webster except those used to determine the effect of a forced swim (panel A) or injection of BRL (B) in C57BL/6J mice not expressing the UCP1 synthetic gene (KO) or UCP1 replete controls (WT). Grip force values were taken immediately (within 5 min) after forced swims that lasted for 15 min in water maintained at 26°C. BRL37344 was injected s.c. at a dose of 1 mg/kg in Swiss Webster mice and 10 mg/kg in UCP1-KO and WT C57BL/6J mice. Brown adipose tissue was chemically ablated by injection of Rose Bengal combined with high intensity illumination of the interscapular brown adipose depot two weeks prior to testing. Control mice were also injected with Rose Bengal but not illuminated. Grip force values were taken 24 hr after the injection of BRL37344 in ablated/sham mice and 60 min after the injection BRL67344 in the WT and UCP1-KO mice. In all figures, asterisks (*) depict statistically significant differences between the groups indicated ($P<0.05$) using a two-tailed unpaired Student's t-test to compare grip force responses. Hashtags (#) designate groups that differed significantly ($P<0.05$) from their own baseline control values taken on the same day when evaluated using a two-tailed, paired Student's t-test. The number of mice/group is indicated at the base of each column.

Figure 2

Effect of a daily forced swim on mean (±SEM) grip force responses, body temperature and interscapular brown adipose tissue (BAT) weight. The forced swim lasted for 15 min in water maintained at 26°C. Grip force values (A) and rectal temperature (B) were taken immediately

(within 5 min) after each daily swim and compared to mice not subjected to a swim stress. The weight of interscapular BAT (C) was measured in both groups 24 h after the final swim. An unpaired, two-way Student's t-test was used to compare the effect of a daily forced swim on grip force responses and body temperature to mice not exposed to the daily swim on the same day of testing. In all figures, asterisks indicate statistically significant differences between treated and control mice when $P < 0.05$. Throughout all figures, SEM is calculated for all points but not shown graphically where it is smaller than the size of the symbol depicting the mean. The number of female mice/group is indicated at the bottom of each column in panel C.

Figure 3

Effect of a daily injection of BRL37344, a β3 adrenergic receptor agonist, on mean (±SEM) grip force responses, body temperature and interscapular brown adipose tissue (BAT) weight. Grip force values (A and B) and rectal temperature (C) were taken 60 min (A) or 24 h (B) after each daily swim and compared to mice injected with vehicle. Rectal temperature was taken 15 min and 24 h after each injection (C). The weight of interscapular BAT (D) was measured in both groups 24 h after the final daily s.c. injection of 1 mg/kg of BRL37344. An unpaired, two-way Student's t-test was used to compare the effect of BRL37344 to that of vehicle on daily grip force responses, body temperature 15 min after injection, body temperature 24 h after injection, and BAT weight. Asterisks indicate statistically significant differences between groups when $P < 0.05$. SEM is calculated for all points but not shown graphically where it is smaller than the size of the symbol depicting the mean. The number of female mice/group is indicated at the bottom of each column in panel D.

Figure 4

Effect of morphine on the mean (±SEM) decrease in grip force responses induced by BRL37344. Mice were injected s.c. with either vehicle or 1 mg/kg daily for 3 days, 3 mg/kg daily for 3 days, and 10 mg/kg of BRL37344 daily for 2 days followed 4 h later by an additional injection i.p. of 10 mg/kg of morphine or saline. The values represent the mean (±SEM) grip force responses before

(Vehicle or BRL37344 only) and 30 min after injection of morphine or saline. Asterisks indicates a significant difference ($P<0.05$) between the treatments indicated as determined using one-way ANOVA followed by the Newman-Keuls post hoc analysis. The number of mice/group is indicated at the bottom of each column.

Figure 5

Comparison of the effect of chemical ablation of interscapular brown adipose on the mean (±SEM) grip force responses and brown adipose tissue weight after each of 14 daily forced swims or 14 daily injections of BRL37344. Two weeks prior to swims or injections, brown adipose tissue was chemically ablated by injection of Rose Bengal combined with high intensity illumination of the BAT depot, as described in detail in methods. Control mice were also injected with Rose Bengal but not illuminated (Sham). Panels A and C reflect data from mice that were subjected to a 15-min forced swim in water maintained at 26°C. Panels B and D reflect data from mice that were injected s.c. daily with 1 mg/kg of BRL37344. Grip force measurements were taken before and 0 min after a forced swim (A) or 60 min after each injection (B). Brown adipose tissue was weighed in mice 24 h after the final swim or final injection of BRL37344. An unpaired, two-way Student's t-test was used to compare the effect of a daily swim or a daily injection in BAT-ablated mice to sham-operated mice tested on the same day. Asterisks indicate statistically significant differences between the two groups of mice when $P <0.05$. The number of male mice/group is indicated at the bottom of each column in panels C and D.

Figure 6

Effect of a daily forced swim on the rectal temperatures of UCP1-KO and wild type (WT) C57BL/6J mice before (Baseline) and immediately after the daily swim and on their body weights throughout the study. Values in panel A reflect mean (±SEM) rectal temperatures at baseline compared to that immediately after a 15-min forced swim (at 26°C). The values in panel B represent the mean (±SEM) body weights when mice arrived (day 1) and at the end of the study (day 33). An unpaired, two-tailed Student's t-test was used to compare temperatures between the two groups. A paired two-tailed Student's t-test was used to compare changes in these values due to swim within each group. Asterisks indicate statistically significant differences between the two groups indicated when $P < 0.05$. The number of female mice/group is indicated at the bottom of each column.

Figure 1

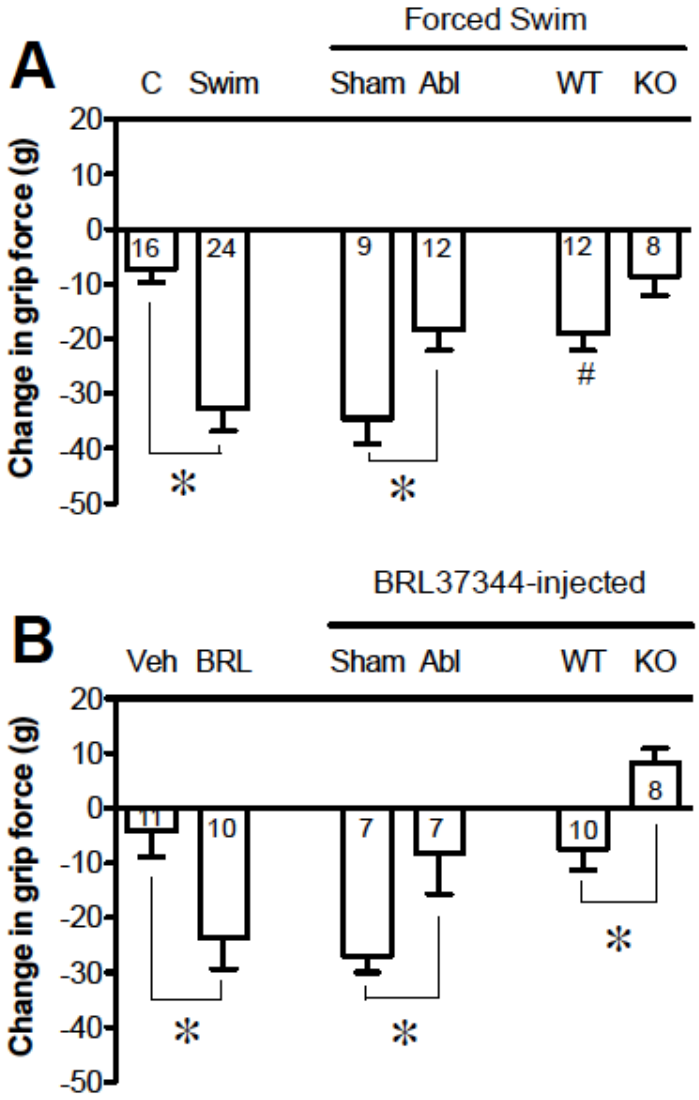

A
Forced Swim
C
Swim
Sham
Abl
WT
KO
Change in grip force (g)
20
10
0
-10
-20
-30
-40
-50
16
24
9
12
12
8
#
*
*
B
BRL37344-injected
Veh
BRL
Sham
Abl
WT
KO
Change in grip force (g)
11
10
7
7
10
8
*
*
*

Figure 2

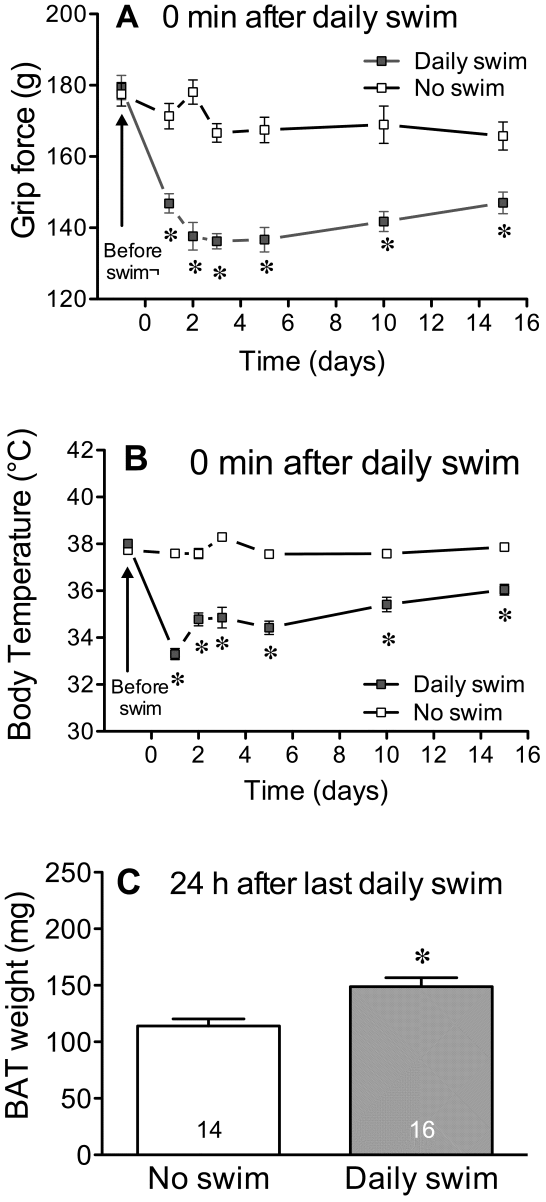

A 0 min after daily swim
Grip force (g)
200
180
160
140
120
Before swim
Daily swim
No swim
0 2 4 6 8 10 12 14 16
Time (days)
B 0 min after daily swim
Body Temperature (°C)
42
40
38
36
34
32
30
Before swim
Daily swim
No swim
0 2 4 6 8 10 12 14 16
Time (days)
C 24 h after last daily swim
BAT weight (mg)
250
200
150
100
50
0
14
16
No swim
Daily swim

Figure 3

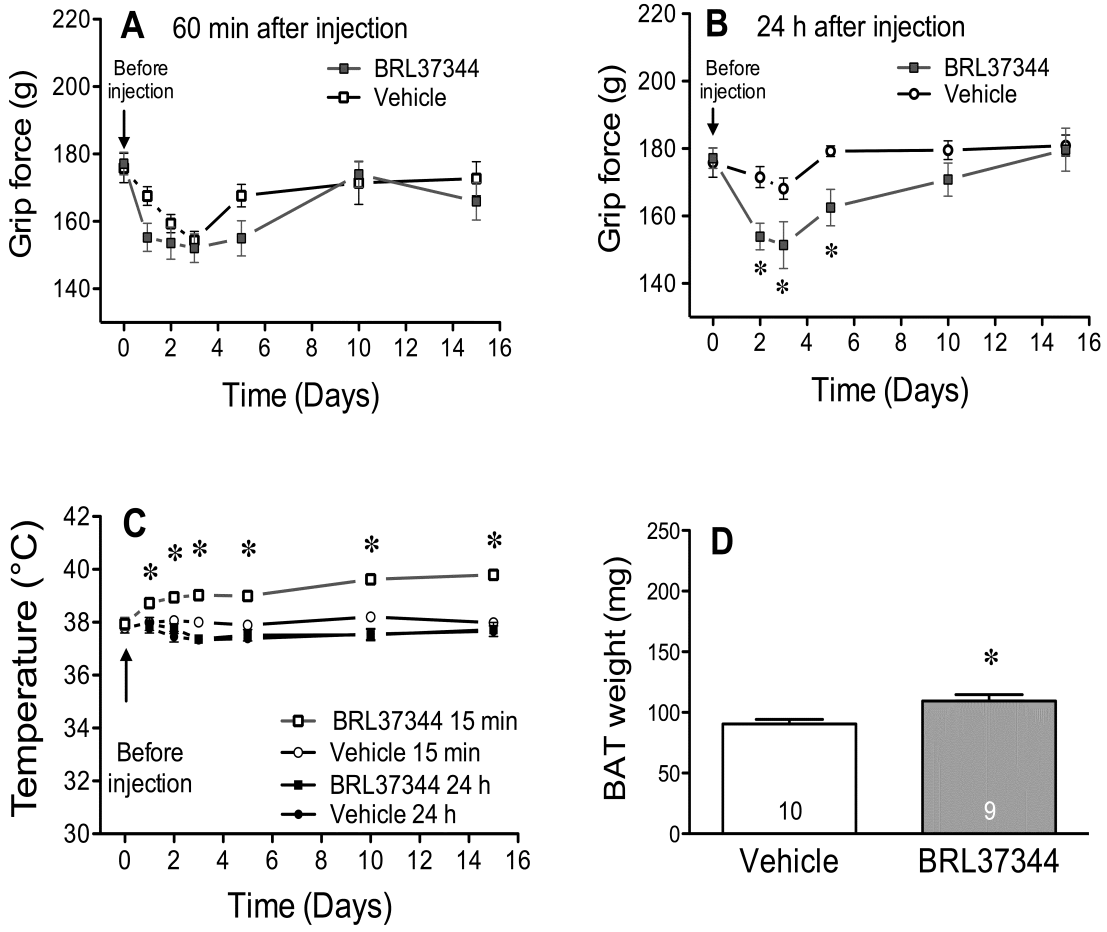

A
60 min after injection
Before injection
BRL37344
Vehicle
Grip force (g)
Time (Days)
B
24 h after injection
Before injection
BRL37344
Vehicle
Grip force (g)
Time (Days)
C
Temperature (°C)
Before injection
BRL37344 15 min
Vehicle 15 min
BRL37344 24 h
Vehicle 24 h
Time (Days)
D
BAT weight (mg)
10
9
Vehicle
BRL37344

Figure 4

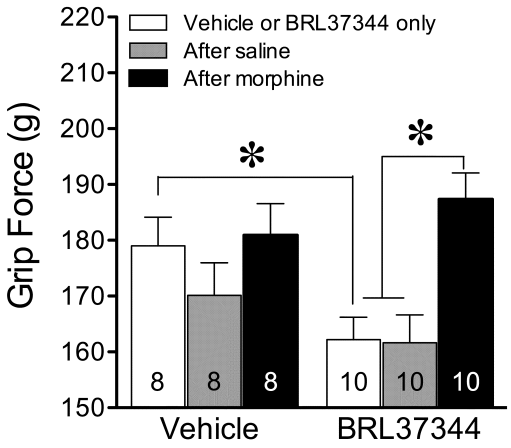

Vehicle or BRL37344 only
After saline
After morphine
Grip Force (g)
220
210
200
190
180
170
160
150
*
*
8
8
8
10
10
10
Vehicle
BRL37344

Figure 5

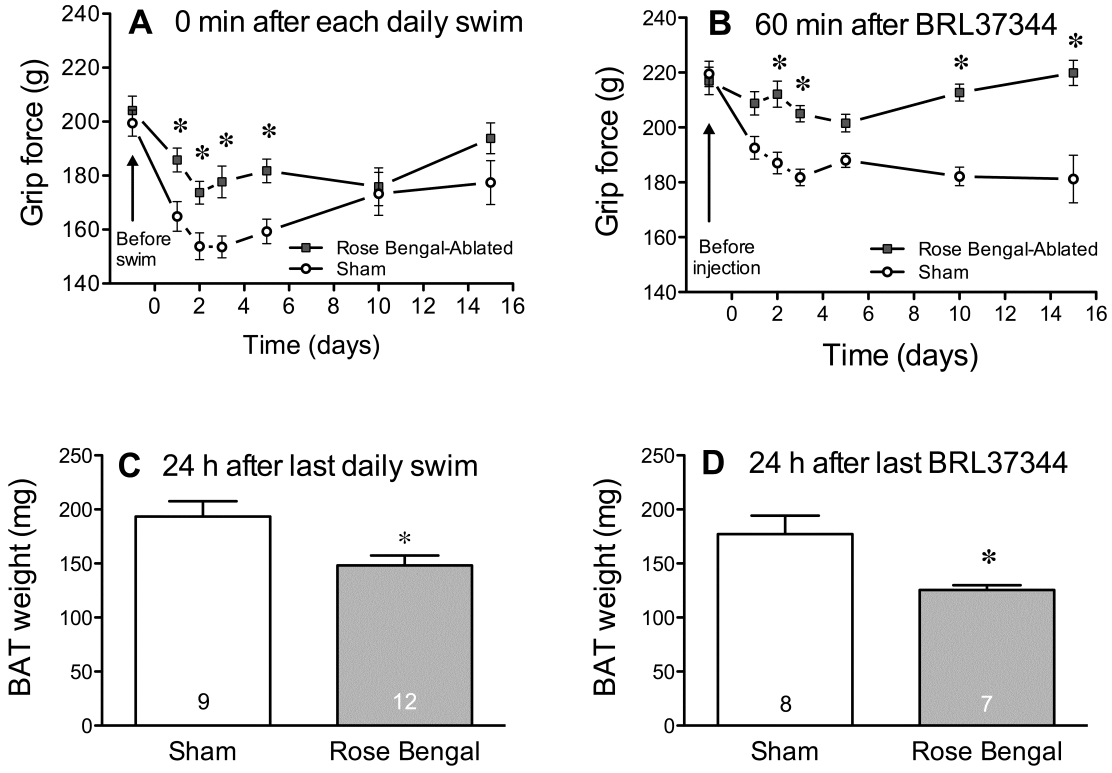
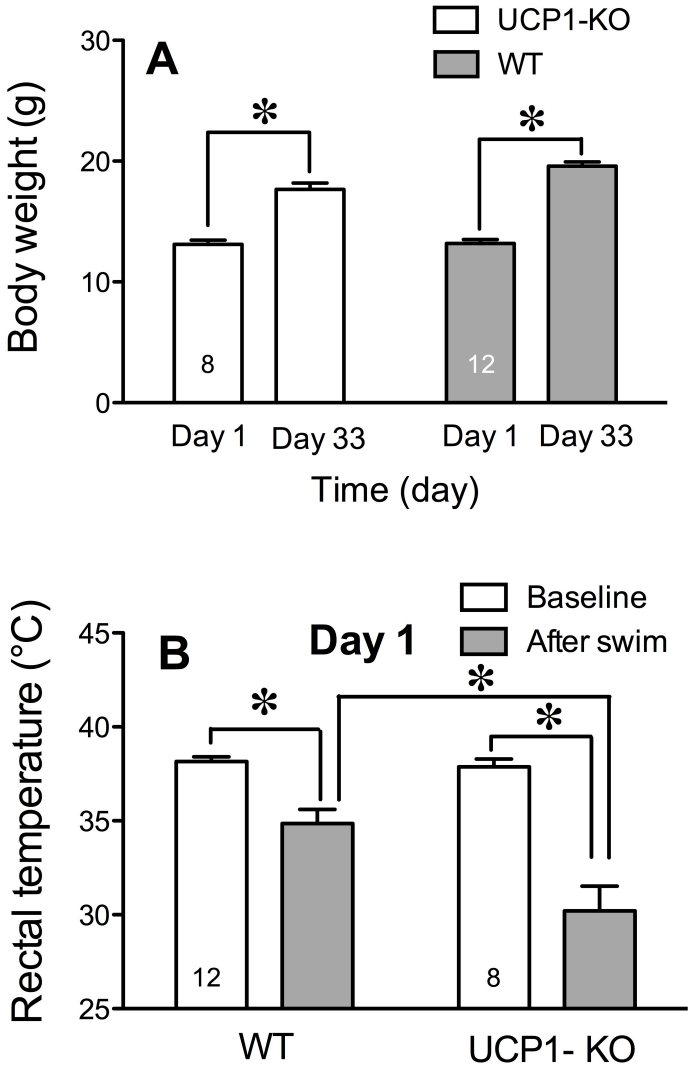

A 0 min after each daily swim
Grip force (g)
Time (days)
Before swim
Rose Bengal-Ablated
Sham
B 60 min after BRL37344
Before injection
C 24 h after last daily swim
BAT weight (mg)
Sham
Rose Bengal
9
12
D 24 h after last BRL37344
8
7

Figure 6


A
UCP1-KO
WT
Body weight (g)
30
20
10
0
8
12
Day 1
Day 33
Day 1
Day 33
Time (day)
B
Day 1
Baseline
After swim
Rectal temperature (°C)
45
40
35
30
25
12
8
WT
UCP1- KO

Bibliography

[1] Abdelhamid RE, Kovacs KJ, Honda CN, Nunez MG, Larson AA. Resiniferatoxin (RTX) causes a uniquely protracted musculoskeletal hyperalgesia in mice by activation of TRPV1 receptors. J Pain 2013;14(12):1629-1641.

[2] Almind K, Manieri M, Sivitz WI, Cinti S, Kahn CR. Ectopic brown adipose tissue in muscle provides a mechanism for differences in risk of metabolic syndrome in mice. Proc Natl Acad Sci U S A 2007;104(7):2366-2371.

[3] Brooks SL, Neville AM, Rothwell NJ, Stock MJ, Wilson S. Sympathetic activation of brown-adipose-tissue thermogenesis in cachexia. Biosci Rep 1981;1(6):509-517.

[4] Bruehl S. An update on the pathophysiology of complex regional pain syndrome. Anesthesiology 2010;113(3):713-725.

[5] Bukowiecki L, Collet AJ, Follea N, Guay G, Jahjah L. Brown adipose tissue hyperplasia: a fundamental mechanism of adaptation to cold and hyperphagia. Am J Physiol 1982;242(6):E353-359.

[6] Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev 2004;84(1):277-359.

[7] Cawthorne MA, Sennitt MV, Arch JR, Smith SA. BRL 35135, a potent and selective atypical beta-adrenoceptor agonist. Am J Clin Nutr 1992;55(1 Suppl):252S-257S.

[8] Coman OA, Paunescu H, Ghita I, Coman L, Badararu A, Fulga I. Beta 3 adrenergic receptors: molecular, histological, functional and pharmacological approaches. Rom J Morphol Embryol 2009;50(2):169-179.

[9] Drummond PD, Finch PM. Persistence of pain induced by startle and forehead cooling after sympathetic blockade in patients with complex regional pain syndrome. J Neurol Neurosurg Psychiatry 2004;75(1):98-102.

[10] Griep EN, Boersma JW, de Kloet ER. Altered reactivity of the hypothalamic-pituitary-adrenal axis in the primary fibromyalgia syndrome. J Rheumatol 1993;20(3):469-474.

[11] Halter JB, Pflug AE, Porte D, Jr. Mechanism of plasma catecholamine increases during surgical stress in man. J Clin Endocrinol Metab 1977;45(5):936-944.

[12] Hayes NL, Rustioni A. Spinothalamic asnd spinomedullary neurons in macaques: a single and double retrograde tracer study. Neuroscience 1980;5(5):861-874.

[13] Himms-Hagen J. Brown adipose tissue metabolism and thermogenesis. Annu Rev Nutr 1985;5:69-94.

[14] Hofmann WE, Liu X, Bearden CM, Harper ME, Kozak LP. Effects of genetic background on thermoregulation and fatty acid-induced uncoupling of mitochondria in UCP1-deficient mice. J Biol Chem 2001;276(15):12460-12465.

[15] Kataoka N, Hioki H, Kaneko T, Nakamura K. Psychological stress activates a dorsomedial hypothalamus-medullary raphe circuit driving brown adipose tissue thermogenesis and hyperthermia. Cell Metab 2014;20(2):346-358.

[16] Kawanishi C, Fukuda M, Tamura R, Nishijo H, Ono T. Effects of repeated cold stress on feeding, avoidance behavior, and pain-related nerve fiber activity. Physiol Behav 1997;62(4):849-855.

[17] Kehl LJ, Kovacs KJ, Larson AA. Tolerance develops to the effect of lipopolysaccharides on movement-evoked hyperalgesia when administered chronically by a systemic but not an intrathecal route. Pain 2004;111(1-2):104-115.

[18] Kehl LJ, Trempe TM, Hargreaves KM. A new animal model for assessing mechanisms and management of muscle hyperalgesia. Pain 2000;85(3):333-343.

[19] Khasar SG, Green PG, Levine JD. Repeated sound stress enhances inflammatory pain in the rat. Pain 2005;116(1-2):79-86.

[20] Krief S, Lonnqvist F, Raimbault S, Baude B, Van Spronsen A, Arner P, Strosberg AD, Ricquier D, Emorine LJ. Tissue distribution of beta 3-adrenergic receptor mRNA in man. J Clin Invest 1993;91(1):344-349.
[21] Kuji I, Imabayashi E, Minagawa A, Matsuda H, Miyauchi T. Brown adipose tissue demonstrating intense FDG uptake in a patient with mediastinal pheochromocytoma. Ann Nucl Med 2008;22(3):231-235.
[22] Larson AA, Pardo JV, Pasley JD. Review of overlap between thermoregulation and pain modulation in fibromyalgia. Clin J Pain 2014;30(6):544-555.
[23] Lofgren M, Norrbrink C. Pain relief in women with fibromyalgia: a cross-over study of superficial warmth stimulation and transcutaneous electrical nerve stimulation. J Rehabil Med 2009;41(7):557-562.
[24] Martinez-Lavin M, Vidal M, Barbosa RE, Pineda C, Casanova JM, Nava A. Norepinephrine-evoked pain in fibromyalgia. A randomized pilot study [ISRCTN70707830]. BMC Musculoskelet Disord 2002;3:2.
[25] Matthias A, Ohlson KB, Fredriksson JM, Jacobsson A, Nedergaard J, Cannon B. Thermogenic responses in brown fat cells are fully UCP1-dependent. UCP2 or UCP3 do not substitute for UCP1 in adrenergically or fatty scid-induced thermogenesis. J Biol Chem 2000;275(33):25073-25081.
[26] McMahon SB. Mechanisms of sympathetic pain. Br Med Bull 1991;47(3):584-600.
[27] Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, Cannon B. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. Biochim Biophys Acta 2001;1504(1):82-106.
[28] Nishiyori M, Ueda H. Prolonged gabapentin analgesia in an experimental mouse model of fibromyalgia. Mol Pain 2008;4:52.
[29] Oriowo MA, Chapman H, Kirkham DM, Sennitt MV, Ruffolo RR, Jr., Cawthorne MA. The selectivity in vitro of the stereoisomers of the beta-3 adrenoceptor agonist BRL 37344. J Pharmacol Exp Ther 1996;277(1):22-27.
[30] Pertovaara A. Noradrenergic pain modulation. Prog Neurobiol 2006;80(2):53-83.
[31] Porsolt RD. Animal model of depression. Biomedicine 1979;30(3):139-140.
[32] Porsolt RD, Bertin A, Blavet N, Deniel M, Jalfre M. Immobility induced by forced swimming in rats: effects of agents which modify central catecholamine and serotonin activity. Eur J Pharmacol 1979;57(2-3):201-210.
[33] Porsolt RD, Deniel M, Jalfre M. Forced swimming in rats: hypothermia, immobility and the effects of imipramine. Eur J Pharmacol 1979;57(4):431-436.
[34] Quintero L, Moreno M, Avila C, Arcaya J, Maixner W, Suarez-Roca H. Long-lasting delayed hyperalgesia after subchronic swim stress. Pharmacol Biochem Behav 2000;67(3):449-458.
[35] Ryu V, Garretson JT, Liu Y, Vaughan CH, Bartness TJ. Brown adipose tissue has sympathetic-sensory feedback circuits. J Neurosci 2015;35(5):2181-2190.
[36] Sarzi-Puttini P, Atzeni F, Diana A, Doria A, Furlan R. Increased neural sympathetic activation in fibromyalgia syndrome. Ann N Y Acad Sci 2006;1069:109-117.
[37] Sinclair DC, Weddell G, Feindel WH. Referred pain and associated phenomena. Brain 1948;71(2):184-211.
[38] Suarez-Roca H, Quintero L, Arcaya JL, Maixner W, Rao SG. Stress-induced muscle and cutaneous hyperalgesia: differential effect of milnacipran. Physiol Behav 2006;88(1-2):82-87.
[39] Suarez-Roca H, Silva JA, Arcaya JL, Quintero L, Maixner W, Pinerua-Shuhaibar L. Role of mu-opioid and NMDA receptors in the development and maintenance of repeated swim stress-induced thermal hyperalgesia. Behav Brain Res 2006;167(2):205-211.
[40] Vallbo AB, Hagbarth KE, Torebjork HE, Wallin BG. Somatosensory, proprioceptive, and sympathetic activity in human peripheral nerves. Physiol Rev 1979;59(4):919-957.

[41] Yoneshiro T, Aita S, Kawai Y, Iwanaga T, Saito M. Nonpungent capsaicin analogs (capsinoids) increase energy expenditure through the activation of brown adipose tissue in humans. Am J Clin Nutr 2012;95(4):845-850.
[42] Zhang S, Kluge B, Huang F, Nordstrom T, Doolen S, Gross M, Sarmiere P, Holmberg EG. Photochemical scar ablation in chronically contused spinal cord of rat. J Neurotrauma 2007;24(2):411-420.