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ORIGINAL RESEARCH

Sound-stress-induced altered nociceptive behaviors are associated with increased spinal CRFR2 gene expression in a rat model of burn injury

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Abstract: Sound stress (SS) elicits behavioral changes, including pain behaviors. However, the neuronal mechanisms underlying SS-induced pain behaviors remain to be explored. The current study examined the effects of SS on nociceptive behaviors and changes in expression of the spinal corticotropin-releasing factor (CRF) system in male Sprague Dawley rats with and without thermal pain. We also studied the effects of SS on plasma corticosterone and fecal output. Rats were exposed to 3 days of SS protocol (n = 12/group). Changes in nociceptive behaviors were assessed using thermal and mechanical pain tests. Following the induction of SS, a subgroup of rats (n = 6/group) was inflicted with thermal injury and on day 14 postburn nociceptive behaviors were reassessed. Spinal CRF receptor mRNA expression was analyzed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR). In addition, plasma corticosterone and spinal CRF concentrations were quantified using enzyme-linked immunosorbent assay (ELISA). Increased defecation was observed in SS rats. SS produced transient mechanical allodynia in naive rats, whereas it exacerbated thermal pain in thermally injured rats. Spinal *CRFR2* mRNA expression was unaffected by stress or thermal injury alone, but their combined effect significantly increased its expression. SS had no effect on plasma corticosterone and spinal CRF protein in postburn rats. To conclude, SS is capable of exacerbating postburn thermal pain, which is linked to increased *CRFR2* gene expression in the spinal cord. Future studies have to delineate whether attenuation of CRFR2 signaling at the spinal level prevents stress-induced exacerbation of burn pain.

Keywords: sound stress, corticotropin-releasing factor receptor-2, thermal injury, corticosterone, thermal pain, mechanical pain

Introduction

Stress alters pain and analgesia signaling pathways, and the phenomena as such are known as stress-induced hyperalgesia (SIH) and stress-induced analgesia (SIA). Numerous stressors can produce SIH and SIA in rodents depending on the nature, duration and intensity of stressor(s).[1–3] Sound is a vibration of the surrounding air or another medium that is sensed in the ear. It can induce pleasant or unpleasant sensation, and it depends on magnitude and duration of exposure to sound. Noise is an unpleasant form of sound. Exposure to noise of low frequency or loud noise causes auditory deficit.[4,5] Of note, sound or noise is one of the potent psychological stressors that can affect our health.[6,7] The exposure to sound stress (SS) is common in the battlefield, in urban traffic and also in some work environments. In addition to exposure to SS, the

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occurrence of burn injuries is highly likely in members serving in the armed forces and the fire department. Patients with burn injury experience intense pain, and in some cases, the pain worsens and is persistent for years post injury. Unfortunately, the currently available therapies are inadequate to manage burn pain. At least one of the reasons for this might be attributed to our poor understanding of the nature of burn pain. Consistent studies have shown that exposure to environmental stressors prior to injury can influence different types of pain state such as postsurgical pain,[8] muscle pain and visceral pain.[9] However, the effects of SS on postburn pain have not been previously explored.

By and large, stressors influence nociception and antinociception through the modulation of corticotropin-releasing factor (CRF) system.[10,11] CRF and CRF receptors (CRFR1 and CRFR2) are widely distributed in the central nervous system (CNS).[12,13] The apparent involvement of CRF-CRFR1 and CRF-CRFR2 signaling in nociception, antinociception, SIH and SIA depends on some specific aspects such as experimental condition (the animal model of injury or disease) studied and areas of the CNS examined.[3,11,14–17] However, there have been no reports on the impact of SS on spinal CRF system.

Full-thickness thermal injury (FTTI) to the plantar surface of the rat hind paw produces mechanical allodynia and thermal hyperalgesia. The nociceptive behaviors develop within 24 h of injury and continue until day 14 and by day 21 subside.[18,19] There is one report which demonstrates that the exposure of rats to forced swim stress before inflicting thermal injury aggravates mechanical allodynia and thermal hyperalgesia in rats.[20] However, the studies on the effect of SS on pain behaviors in FTTI rats and the underlying mechanism are scanty. With a view to addressing these gaps, we primarily examined the changes in nociceptive behaviors in both uninjured and thermally injured rats prior exposed to SS. In addition, we examined the impact of SS and thermal pain on the expression of CRF and CRF receptors in the lumbar spinal cord.

Materials and methods

Animals

We used 24 adult male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA) of 7–8 weeks old for this study. Rats were pair housed on a 12 h light/dark cycle (6 am–6 pm) with ad libitum access to rodent chow and water. The rats were subjected to experiments after they spent 1 week in the vivarium. Before exposure to procedures, the rat body weight was 260–280 g, and it reached 360–380 g by the end of the experiment. The US Army Institute of Surgical Research (USAISR) Institutional Animal Care and Use Committee (IACUC) approved all experimental protocols. This study was conducted in compliance with the Animal Welfare Act, by implementing Animal Welfare Regulations and the principles of the Guide for the Care and Use of Laboratory animals. All measures were undertaken to minimize the number of animals to be used for this study.

SS protocol

SS was induced using the Startle Response System apparatus (SR-Labs, San Diego Instruments, San Diego, CA, USA; model numbers SIC002650–SIC002655), utilizing the programmable SR-Lab's software. The apparatus consists of an acrylic enclosure (8″ [length] × 3 1⁄2″) contained in an acrylonitrile butadiene styrene (ABS) isolation chamber. Rats were exposed to SS as described previously by Khasar et al[21] with slight modifications. Briefly, rats were first acclimatized to the testing chamber for 50 min for 1 day before experiments. On experimental day, rats were again habituated to the test chamber for 20 min followed by exposure to 105 dB tone with frequencies ranging from 11 to 19 kHz, each lasting for 5–10 s randomly each minute over a total 30-min period. This procedure was performed repeatedly for 3 consecutive days. Control rats (no stress [NS] group) were placed in the same testing chamber for 50 min for 4 successive days but without exposure to the sound stimulus. Animals were returned to their home cages after sound or sham SS procedure.

Induction of thermal injury

Unilateral FTTI was induced in deeply anesthetized (3–4% isoflurane in oxygen) rats by placing a preheated (100°C) soldering tip on the mid-plantar surface of the right hind paw for 30 s.[18] The post-injury care was performed according to our IACUC recommendations. Briefly, immediately after induction of thermal injury, silver sulfoxide was applied one time to the site of injury to minimize infection. In addition, animals' general appearance and wound assessment were monitored throughout the experimental period. As the focus of this study was to measure pain behaviors, no analgesia was administered.

Thermal nociceptive test

Rats were examined for thermal nociception as described previously.[22] Briefly, the test was performed using analgesia instrument (Model 390; IITC Life Science, Woodland Hills, CA, USA). After acclimatizing the rats to the behavioral

room (30 min) and to the Plexiglas chambers (20 min), the radiant light beam was focused on the adjacent, proximal area to the injury site of the hind paw until the animal voluntarily removed its paw. The time between the application of thermal stimuli and response time was recorded as the paw withdrawal latency (PWL). The intensity of the beam was set to 40% to produce baseline PWL of ~10–12 s in naive rats. A cutoff of 20 s was applied to avoid tissue damage. Three trials for each hind paw, with an interval of 5 min, were averaged and used for the analysis. To study the post-SS effects on PWL in uninjured rats (at 30 min, 1 and 14 days post stress) scores from both left and right paws were combined to yield the mean PWL. In thermally injured rats, the PWLs of ipsilateral and contralateral paws from respective experimental groups were compared. ΔPWL was calculated as shown elsewhere: ΔPWL = tested PWL – baseline PWL.[22–24]

Mechanosensitivity assay

Rats were first habituated to the behavioral testing room for 30 min followed by placement on a metal grid in Plexiglas chambers for 20 min. To examine mechanosensitivity in response to a non-noxious stimulus, the Dynamic Plantar Anesthesiometer (Ugo Basile, Collegeville, PA, USA) was used to measure the force (in grams) required to elicit a voluntary withdrawal of the hind paw from a rigid von Frey tip. This was recorded as paw withdrawal threshold (PWT; g). The stimulus was presented at the adjacent, proximal site of thermal injury. Each rat was tested on both the right and left hind paw for three times, and the average was taken. As stated under the thermal nociceptive test, the PWTs from the left and right paws of the uninjured rats were combined to yield the mean PWT, whereas in the thermally injured state the PWT was recorded from the ipsilateral paw (injured) and contralateral paw was compared with their respective control group. ΔPWT was calculated as ΔPWT = tested PWT – baseline PWT.[23,24] A 30 min time interval was maintained between thermal and mechanical testing.

Blood sample collection

Unanesthetized rats were euthanized by decapitation, and trunk blood was immediately collected in microtainer tubes containing EDTA (BD 365974) within 2 h (between 12 and 2 pm) after the final behavioral tests[25] (day 18). Tubes were inverted 10 times for thorough mixing. All blood samples were centrifuged 15 min at 1000 ×*g* at 4°C within 30 min of collection. The plasma supernatant was then transferred to a clean microfuge tube and stored until analyzed by enzyme-linked immunosorbent assay (ELISA).

Total RNA isolation

Following decapitation, the L4–L6 region of the spinal cord was dissected and immediately frozen in liquid nitrogen. Total RNA from the L4–L6 region of the spinal cord was isolated. Briefly, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)-based (20 mM HEPES; 1 mM EDTA; 40 U/mL RNAse inhibitor) buffer was added to the spinal cord samples and homogenized two times for 20 s, centrifuged at 14,000 ×*g* for 20 min at 4°C. The pellet was then resuspended in Tri reagent, and RNA was isolated using the Zymogen DirectZol RNA Miniprep Kit (ZRC175939). RNA concentration was determined by Nanodrop instrument. We used hypothalamus as a control tissue and processed similarly to extract total RNA.

ELISA analysis

CRF or corticosterone concentrations were determined by ELISA (LSbio LS-F5619 and LS-F25811) following the manufacturer's directions. Briefly, standards or samples were incubated with detection reagent A (biotin-conjugated target antigen) for 1 h at 37°C in a plate pre-coated with target-specific capture antibody. The antigens in the standards or samples compete with the biotin-conjugated antigen to bind to the capture antibody. Incubation with detection reagent B (avidin–horseradish peroxidase [HRP] conjugate) for 30 min at 37°C was performed followed by washing five times. Samples were then incubated for 15 min at 37°C with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution followed by equal incubation time with stop solution. The TMB substrate reacts with the HRP enzyme resulting in color development. The optical density was determined using a microplate reader at 450 nm wavelength.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Reverse transcriptase was performed using the iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA; Cat#: 1708890) following the manufacturer's directions. PCR was performed using iQ Sybr Green supermix (Bio-Rad Laboratories Inc.; 170-8880). The following PCR primers were used: *CRFR1* forward: TGC CTG AGA AAC ATC ATC CAC TGG; *CRFR1* reverse: TAA TTG TAG GCG GCT GTC ACC AAC; *CRFR2* forward: AAC GGC ATC AAG TAC AAC ACG ACA; *CRFR2* reverse: CGA TTC GGT AAT GCA GGT CAT AC. These primers were adapted from LaBerge et al.[26,27] All primers were optimized prior to run-

ning the experimental samples. The amplified PCR products were run on a 1% agarose gel precast with ethidium bromide (Bio-Rad Laboratories Inc.; Cat#: 1613022) and visualized with ultraviolet (UV) light (ProteinSimple Fluorchem Q). Integrated density was determined for each sample using Image J analysis.[26,28,29]

Experimental design and procedure

Figure 1 shows the scheme of experiments. All experiments were performed in a blinded fashion.

Experiment 1: effect of SS on defecation and nociceptive thresholds in uninjured rats

Two groups of rats were used. Group 1 (n = 12) served as NS control and group 2 (n=12) as SS. Rats from both groups were first habituated to the testing chamber, and SS was induced as described in the SS procedure.

The total number of fecal pellets defecated on day 1 and day 3 following exposure to SS was averaged and used for analysis. Before induction of SS (baseline) and at 30 min and 24 h after exposure to stress, the rats were tested for changes in nociceptive threshold using thermal and mechanical tests.

Experiment 2: effect of prior SS on nociceptive behaviors in uninjured and thermally injured rats

This experiment was conducted on the same rats used in experiment 1. NS rats from experiment 1 were divided into two groups: group 1 as NS and no injury (NS, n = 6) and group 2 as NS with injury (NS + injury, n = 6). SS rats from experiment 1 were grouped as stress without the injury group (SS, n =6) and stress with the injury group (SS + injury, n = 6). Twenty four h after SS procedure, the basal response to thermal and mechanical stimuli was taken and the right hind paw was inflicted with thermal injury to rats from the NS + injury and SS + injury groups. After 14 days of post-injury and post-stress, the changes in sensitivity to thermal and mechanical stimulus were assessed.

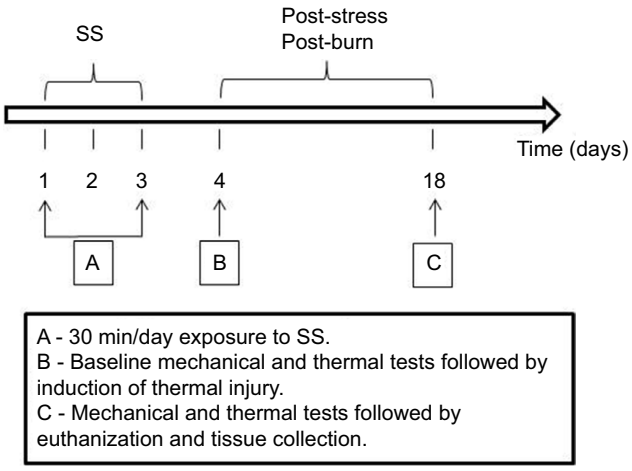


Figure 1 Schematic outline of the experimental design and timeline.
Abbreviation: SS, sound stress.

Experiment 3: effect of prior SS on blood corticosterone, spinal and plasma CRF proteins and CRF receptor mRNA expression

After final behavioral experiments on day 14 post burn and stress, rats from experiment 2 were decapitated and within a minute trunk blood was collected (1 mL), and the samples were analyzed for corticosterone levels using ELISA. After blood collection, the lumbar spinal cord section (L4–L6) and the control tissue, hypothalamus, were harvested and stored at –80°C until used for CRF protein, *CRFR1* and *CRFR2* mRNA studies. CRF expression level was measured using ELISA, whereas *CRF* receptor mRNA transcripts were studied using semiquantitative RT-PCR.

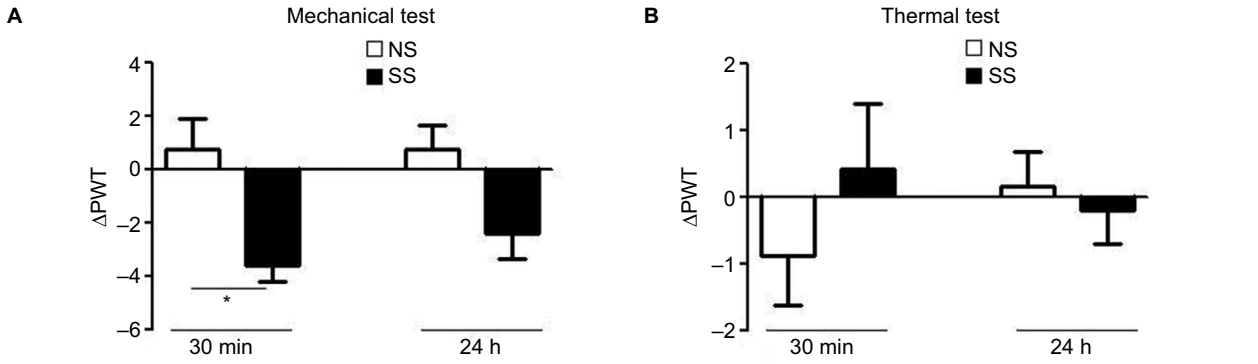
Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 statistics programs (GraphPad Software, Inc., La Jolla, CA, USA). All data were presented as mean ± standard error of the mean (SEM). Two-way repeated-measure ANOVA was utilized to analyze body weight and behavioral changes over a period of time (stress × time interaction). Data from the RT-PCR and ELISA were analyzed using one-way ANOVA. Bonferroni or Newman–Keuls post hoc tests were performed to clarify group differences, as needed. Paired *t*-test was used to compare mean difference of fecal counts between day 1 and day 3, and Student's *t*-test was used to compare fecal output between the NS and SS groups. *P*-values of <0.05 were considered significant.

Results

Effect of SS on basal nociception in uninjured rats

Uninjured rats were tested for changes in sensitivity to thermal and mechanical stimuli at 30 min and 24 h after exposure to SS. In the mechanical pain test, a significant decrease in ΔPWT was observed in SS rats at 30 min ($P<0.05$) but not at 24 h ($P>0.05$) when compared to the NS control group (Figure 2A). No changes in ΔPWL were found between the SS and NS groups at 30 min ($P>0.05$) and 24 h ($P>0.05$) post stress in the thermal test (Figure 2B). Similar results were also noted when absolute values from mechanical and thermal tests were analyzed (Figure 2C, D).



C

	Mean PWT (g)		
	BL	30 min post stress	24 h post stress
NS	25.55 ± 0.88	26.61 ± 1.12	26.30 ± 1.13
SS	27.11 ± 0.52	23.71 ± 0.66 *	24.30 ± 0.98

D

	Mean PWL (s)		
	BL	30 min post stress	24 h post stress
NS	9.12 ± 0.27	8.49 ± 1.61	9.28 ± 0.57
SS	9.14 ± 0.41	9.95 ± 0.55	8.95 ± 0.65

Figure 2 Effect of SS on basal paw withdrawal responses in rats to mechanical and thermal stimuli.
Notes: SS for 30 min for 3 consecutive days markedly reduced the responses to mechanical stimulus in uninjured rats (**A** and **C**). No significant change in basal response between NS and SS was observed following application of the thermal stimulus (**B** and **D**). n = 12/group. Values represent mean ± SEM. *P<0.05.
Abbreviations: BL, baseline; NS, no stress; PWL, paw withdrawal latency; SEM, standard error of the mean; SS, sound stress.

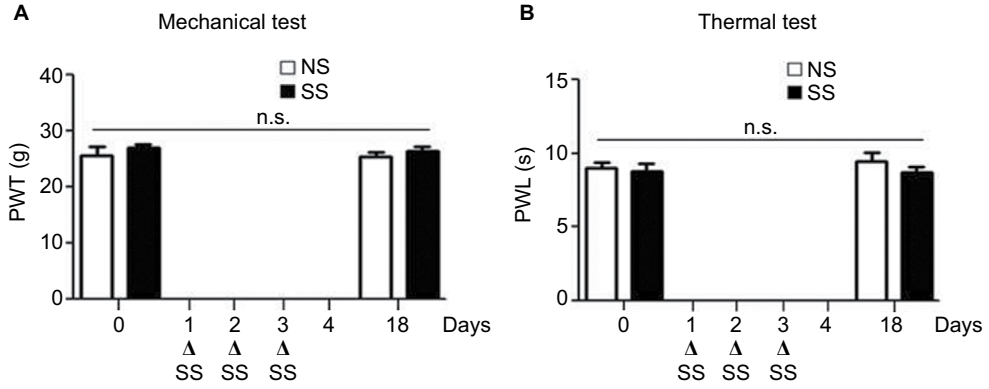


Figure 3 Changes in mechanical and thermal sensitivity in uninjured post-stressed rats.
Notes: At day 14 post stress, there was no significant difference in PWT (**A**) and PWL (**B**) between non-stressed and SS rats. n = 6/group. Values represent mean ± SEM.
Abbreviations: n.s., nonsignificant; NS, no stress; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; SEM, standard error of the mean; SS, sound stress.

Changes in mechanical and thermal sensitivity in uninjured rats on day 14 post SS

As illustrated in Figure 3A and B, on day 14 post SS, the NS and SS groups showed no significant changes in PWT (P>0.05) and PWL (P>0.05) when compared to their respective baseline thresholds.

SS induces enhanced thermal allodynia in thermally injured rats

Consistent with a previous study,[18] on day 14 following thermal injury, the ipsilateral paw of rats from the NS + injury and SS + injury groups showed significantly lower PWL compared to their respective baseline values (P<0.001) indicating the presence of thermal allodynia (Figure 4A). However, rats from the SS + injury group showed increased sensitivity to thermal stimulus compared to the NS + injury group (P<0.05) suggesting that pre-exposure of SS had heightened the magnitude of thermal allodynia (Figure 4A). The PWLs of the contralateral paw of rats from the NS + injury and SS + injury groups did not differ in basal values (P>0.05) and also on day 14 post burn (P>0.05, Figure 4B).

Effect of SS on mechanical allodynia in thermally injured rats

As shown in Figure 5A, mechanical allodynia was developed in rats from the NS + injury and SS + injury groups.

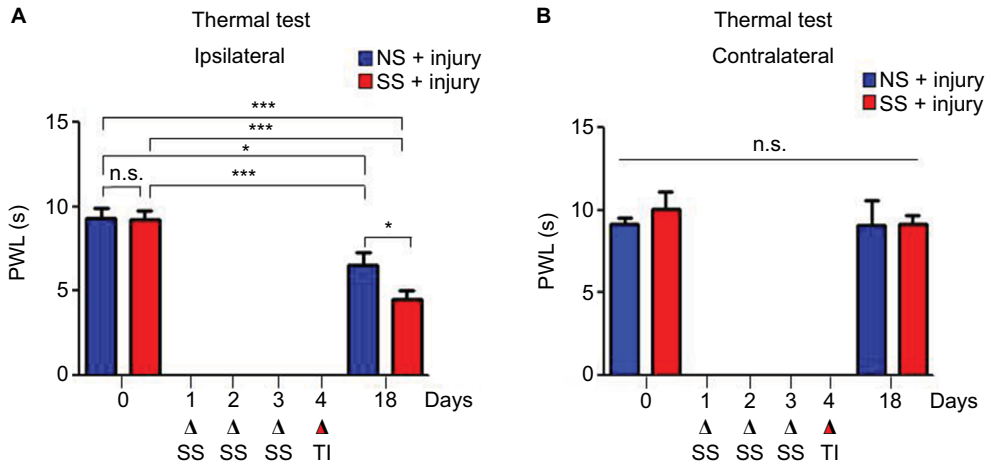


Figure 4 SS exacerbates thermal allodynia in thermally injured rats.
Notes: Compared to their baseline values, a significant decrease in PWL was observed on day 14 post burn and stress in thermally injured rats with or without prior exposure to SS demonstrating the presence of thermal allodynia. However, SS thermally injured rats showed significantly lower PWL than unstressed thermally injured rats indicating the presence of enhanced thermal allodynia (**A**). Either thermal injury or SS and injury combination had any effect on contralateral PWL on day 14 post burn and stress (**B**). n = 6/group. Values represent mean ± SEM. ***P<0.001; *P<0.05.
Abbreviations: n.s., nonsignificant; NS, no stress; PWL, paw withdrawal latency; SEM, standard error of the mean; SS, sound stress; TI, thermal injury.

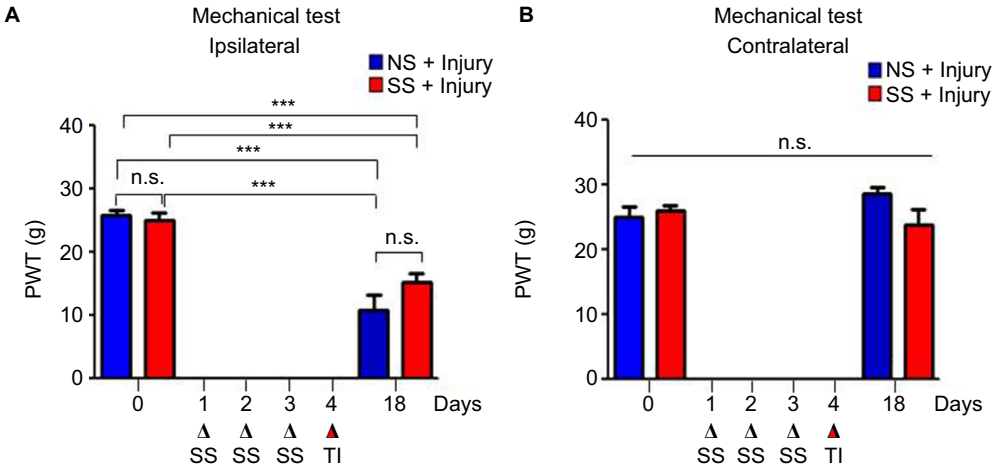


Figure 5 Effects of SS on mechanical nociception in thermally injured rats.
Notes: Compared to their baseline values, thermally injured rats with or without prior exposure to SS showed a significant reduction in PWT on day 14 post burn and stress indicating the presence of mechanical allodynia. However, no remarkable change in PWT was observed between them on day 14 post burn and stress (**A**). Contralateral paw of thermally injured rats with or without prior exposure to SS showed similar PWT. n = 6/group (**B**). Values represent mean ± SEM. ***P<0.001.
Abbreviations: n.s., nonsignificant; PWT, paw withdrawal threshold; SEM, standard error of the mean; SS, sound stress; TI, thermal injury.

Compared to respective baseline PWT, day 14 PWT of the ipsilateral paw (injured) was significantly reduced in rats from the NS + injury and SS + injury groups (P<0.001). However, on day 14 post stress and burn no significant difference in PWT was observed between the NS + injury and SS+ injury groups (P>0.05). Further, when compared, the PWTs of the contralateral paws from the NS + injury and SS + injury groups were almost similar on day 14 (P>0.05, Figure 5B).

Effect of SS and thermal injury on the expression of *CRFR1* and *CRFR2* mRNA levels in the spinal cord

CRFR2 mRNA expression was significantly elevated in the spinal cord of SS + injury rats in comparison to NS, SS and NS + injury rats (P<0.05, Figure 6A and B). On the other hand, *CRFR1* was indeed undetectable in the spinal cord irrespective of stress or injury as evident from the samples analyzed (Figure 6A). We confirmed this analysis by including a positive control tissue (hypothalamus), which showed the expression of both *CRFR1* and *CRFR2* mRNA transcripts.

Effect of SS and thermal injury on CRF and corticosterone levels in the blood plasma and in the spinal cord

The basal CRF level (NS group) was high in plasma (>500 pg/mL, Figure 7A) compared to the spinal cord (<200 pg/mL, Figure 7B). Neither SS and thermal injury nor

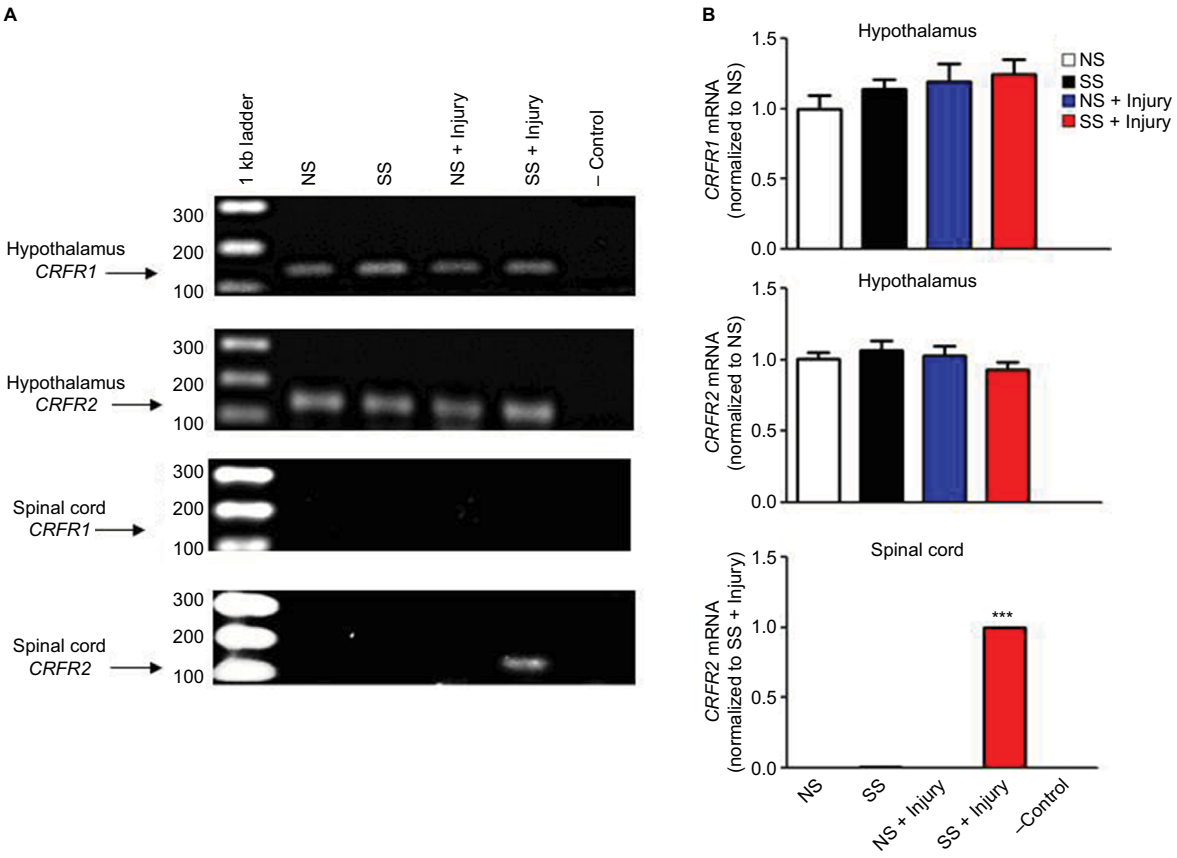


Figure 6 SS upregulates *CRFR2* mRNA expression levels in the lumbar section of the spinal cord of thermally injured rats.
Notes: Semiquantitative RT-PCR analysis showed a significant increase in *CRFR2* mRNA expression only in the thermally injured rats with prior exposure to SS (**A** and **B**). SS group or thermal injury (NS + injury group) alone failed to elicit *CRFR2* mRNA expression. In contrast, *CRFR1* was not expressed in the spinal cord of any of the experimental groups analyzed. Control tissue sample, hypothalamus, shows the expression of CRFR1 and CRFR2 (**A** and **B**). n = 6/group. Values represent mean ± SEM. ***$P<0.05$.
Abbreviations: *CRFR1*, corticotropin-releasing factor receptor-1; *CRFR2*, corticotropin-releasing factor receptor-2; NS, no stress; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean; SS, sound stress.

the composite effect of SS and thermal injury showed any comparable changes in the CRF levels in the plasma ($P>0.05$, Figure 7A) and also in the spinal cord ($P>0.05$, Figure 7B). The plasma corticosterone was detectable in all of the four experimental groups, but no significant difference among the NS, SS, NS + injury and SS + injury groups was observed ($P>0.05$; Figure 7C).

Effect of SS on defecation in uninjured rats

We counted the number of fecal pellets excreted in the SS testing chamber by NS and SS rats at two time points (day 1 and day 3) following exposure to SS and found no significant difference between groups on day 1 ($P>0.05$) and also on day 3 ($P>0.05$). However, as illustrated in Figure 8A, the SS rats showed greater defecation on day 3 compared to day 1 ($P<0.05$), but such changes were not observed in NS rats ($P>0.05$).

Body weight assessment

Next, we studied the effect of SS on body weight gain in uninjured rats during a 3-day period of stress procedure and also we continued to monitor the changes following induction of thermal injury. Rats from all groups showed a steady gain in body weight without significant difference among the NS, SS, NS + injury and SS + injury groups until the end of the experiment ($P>0.05$, Figure 8B).

Discussion

Our results indicated that SS is capable of altering the basal response to non-noxious mechanical stimulus in uninjured rats and also to exacerbate thermal pain in thermally injured rats. SS also increased the spinal *CRFR2* gene expression in thermally injured rats. In addition, SS altered defecation rate in naive animals. The spinal CRF protein concentration, plasma corticosterone levels and body weight were unaffected by SS. Overall, the main findings indicate that SS affects

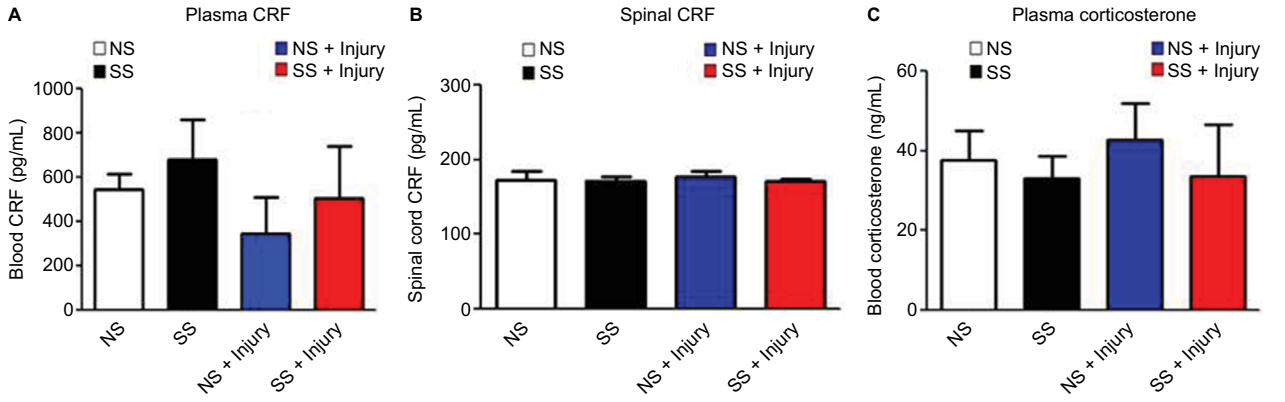


Figure 7 Effects of SS on CRF and corticosterone levels.
Notes: Uninjured and thermally injured rats with or without prior exposure to SS showed comparable levels of CRF protein concentration in the plasma (**A**) and in the spinal cord (**B**). Plasma corticosterone levels also did not vary among experimental groups (**C**). n = 6/group. Values represent mean ± SEM.
Abbreviations: CRF, corticotropin-releasing factor; NS, no stress; SEM, standard error of the mean; SS, sound stress.

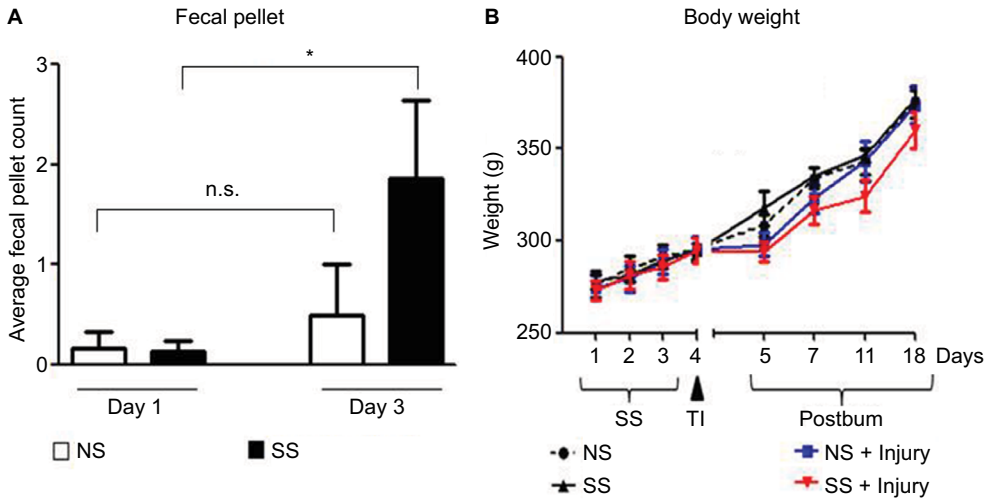


Figure 8 Effects of SS on defecation and body weight.
Notes: No significant difference in fecal pellet counts between NS and SS rats was observed. However, compared to day 1, on day 3 higher rate of defecation was observed in SS rats. n = 12/group (**A**). Uninjured and thermally injured rats with or without prior exposure to SS showed a steady increase in body weight, and there were no concurrent changes among them at the same time of assessments. n = 6/group (**B**). Values represent mean ± SEM. *$P<0.05$.
Abbreviations: n.s., nonsignificant; NS, no stress; SEM, standard error of the mean; SS, sound stress; TI, thermal injury.

post-burn pain and it is associated with changes in *CRFR2* gene expression at the spinal level.

Reports show that exposure to stressful stimuli alters basal pain sensitivity. Our data showed that 3 days of exposure to SS can increase sensitivity to non-noxious mechanical stimulus but not to a thermal stimulus in naive rats. The change in basal sensitivity to the mechanical stimulus was short-lived, up to 24 h post stress. Reassessment of nociceptive behaviors after 14 days of exposure to SS failed to show any changes in mechanical and thermal sensitivity, thus indicating that SS-induced mechanical allodynia in uninjured rats is transient. Although the distinct mechanism of this momentary change in mechanical sensitivity in SS rats remains obscure, it is possible that this type of stress regimen did induce mild anxiety or fear-associated nociception. Indeed, one report indicates that mild non-noxious anxiogenic vibration stress caused hyperalgesia in the tail flick latency test.[30]

Along with previous reports,[18,19] data from the current study showed the presence of mechanical and thermal allodynia on day 14 post injury in both SS and non-stressed rats. Interestingly, SS rats with thermal injury showed enhanced thermal allodynia, but mechanical allodynia was unaffected by SS. Our findings are in agreement with reports by Khasar et al[21,31,32] where they showed that 3 days of exposure to SS exacerbates mechanical hyperalgesia in rats with inflammatory pain. Further, burn injury or SS plus burn injury did not alter thermal or mechanical sensitivity in the contralateral hind paw on day 14 post burn, thus suggesting that the

changes observed on the injured paw were not due to behavioral learning or sensitization effects. Viewed together with previous studies, these findings suggest that SS is a potent psychological stressor that can affect pain sensitivity in the post-injury state.

CRF and its receptors (CRFR1 and CRFR2) play an important role in the modulation of the stress response and pain transmission. The role of CRF receptors in stress-mediated nociceptive transmission is complex: their activation could inhibit or facilitate nociceptive transmission in stressed animals, depending on the distribution of CRFR1 and CRFR2 receptors in the nervous system. For example, a recent study showed that predator odor SIH in rats is mediated by CRF-CRFR1 signaling in the amygdala.[33] Intrathecal administration of NBI-3596, a CRFR1 receptor antagonist, but not Astressin 2B, a CRFR2 receptor antagonist, inhibits tactile hyperalgesia suggesting the important role of CRF-CRFR1signaling in stress-associated nociception at the spinal level.[16] Another report shows CRFR2 antagonist attenuating forced swim-induced musculoskeletal hyperalgesia.[34] Similarly, footshock stress-induced bladder hypersensitivity in rats was blocked at the spinal level by CRFR2 antagonist but not by the CRFR1 antagonist.[9] Our results indicated that SS-induced exacerbated thermal sensitivity in the postburn state is associated with upregulation of *CRFR2* mRNA but not *CRFR1* mRNA in the spinal cord. Even though we did not investigate the effect of the CRFR2 antagonist on SS-induced enhanced thermal allodynia in the current study, our findings of increased expression of *CRFR2* mRNA expression suggest the augmented activity of CRFR2 in the spinal cord. This finding is in line with Robbins and Ness's[9] and Abdelhamid et al's[34] reports, thus substantiating the involvement of spinal CRFR2 in stress-associated nociception. Of note, our data, for the first time, show that either SS or thermal injury alone had no effect on both *CRFR1* and *CRFR2* gene expression in the spinal cord, indicating that the increased expression of *CRFR2* gene in the spinal cord is due to the composite effects of SS and thermal injury**.** Furthermore, the previous study had implicated sympathoadrenal stress axis in SS-mediated exacerbation of mechanical hyperalgesia in inflammatory pain model.[21] Based on the published reports[9,31,34] and the current findings, we can conclude that SS affects nociceptive transmission by acting at multiple sites.

Our data on CRF protein analysis showed a higher concentration of CRF protein in blood plasma (>300 pg/mL) in comparison to spinal cord CRF level (<200 pg/mL) regardless of exposure to SS or thermal injury. However, we found that CRF levels were unaltered in naive and thermally injured rats with or without exposure to SS. It is possible that since CRF binds to CRFR2 with low affinity, it may not be involved in SS-mediated increased thermal pain that we observed in the current study. In addition, it remains to be determined whether urocortin 2, which binds to CRFR2 with high affinity, plays a role in the SS-induced thermal sensitivity in the thermal injury model at the spinal level as studied in a stress-induced urinary bladder hypersensitivity animal model.[9]

Stressful stimulus activates the hypothalamic–pituitary–adrenal (HPA) axis to release corticosterone to control pain sensitivity in stress state.[2] We did not observe significant changes in the plasma corticosterone level between sound-stressed and control rats in naive and thermally injured state. Since we analyzed the corticosterone level on day 14 post stress and post injury, one possibility is that the HPA axis negative feedback mechanism might have contributed to maintaining the basal level of corticosterone.

Stressors also play an important role in the onset and modulation of gut function. It has been shown that rodents exposed to various types of acute and chronic stressors show excessive colonic motility and stimulate defecation.[35,36] To our knowledge, no reports are available on the effect of SS on fecal output. Our data showed no significant changes in defecation between the NS and SS groups on day 1 and day 3 post stress. However, SS rats showed a marked increase in fecal output on day 3 compared to day 1 stressed rats, but not by the control rats. This suggests that repeated exposure to SS resulted in altered colonic motility in stressed rats. There is a possibility that by prolonging SS exposure beyond 3 days may greatly affect the defecation rate than control rats. Earlier studies have implicated CRF-CRFR1 signaling in altered colonic motility and increased defecation in stressed animals.[37,38] Since we did not study the CRF system in the gastrointestinal tract, we cannot conclude whether SS produced any effect on CRF signaling that might have contributed to altered colonic motility in SS rats between day 1 and day 3. Further, defecation but not plasma corticosterone altered suggests that the HPA axis is not mainly involved in SS increased defecation.

Stressors also affect general physiological states in particular body weight. Our results showed that SS and thermal injury or their combined effect had no influence on body weight gain. Comparable findings have also been reported showing that rodents exposed to stress followed by incision injury had no effect on body weight gain.[8] Contradictory results have also been reported showing that animals exposed to chronic mild stress show gradual reduction in

body weight gain when compared to the non-stressed control group.[39] Thus, it is apparent that the nature and duration of the stressor used in experiments have a substantial impact on body weight gain.

Conclusion

The current study illustrates that SS increases postburn thermal allodynia and also augments the expression of *CRFR2* mRNA in the spinal cord of thermally injured rats. SS or thermal injury alone has no effect on the spinal *CRFR2* mRNA. Further studies examining CRFR2 signaling in the spinal cord and, indeed, the contribution of brain CRFR2, in attenuating SS-mediated exacerbation of thermal allodynia in thermally injured state, are warranted.

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Disclosure

The opinions or assertions contained herein are the private views of the authors and are not to be considered as official or as reflecting the views of the Department of the Army or the Department of Defense. The authors report no other conflicts of interest in this work.

References

1. Olango WM, Finn DP. Neurobiology of stress-induced hyperalgesia. *Curr Top Behav Neurosci*. 2014;20:251–280.
2. Butler RK, Finn DP. Stress-induced analgesia. *Prog Neurobiol*. 2009;88(3):184–202.
3. Jennings EM, Okine BN, Roche M, Finn DP. Stress-induced hyperalgesia. *Prog Neurobiol*. 2014;121:1–18.
4. Venet T, Carreres-Pons M, Chalansonnet M, et al. Continuous exposure to low-frequency noise and carbon disulfide: combined effects on hearing. *Neurotoxicology*. 2017;62:151–161.
5. Frenzilli G, Ryskalin L, Ferrucci M, et al. Loud noise exposure produces DNA, neurotransmitter and morphological damage within specific brain areas. *Front Neuroanat*. 2017;11:49.
6. Ising M. [Nocturnal traffic noise and stress hormone elevations in children]. *Schriftenr Ver Wasser Boden Lufthyg*. 2003;112:5–79.
7. Ising H, Michalak R. Stress effects of noise in a field experiment in comparison to reactions to short term noise exposure in the laboratory. *Noise Health*. 2004;6(24):1–7.
8. Wang PK, Cao J, Wang H, et al. Short-term sleep disturbance-induced stress does not affect basal pain perception, but does delay postsurgical pain recovery. *J Pain*. 2015;16(11):1186–1199.
9. Robbins MT, Ness TJ. Footshock-induced urinary bladder hypersensitivity: role of spinal corticotropin-releasing factor receptors. *J Pain*. 2008;9(11):991–998.
10. Vit JP, Clauw DJ, Moallem T, Boudah A, Ohara PT, Jasmin L. Analgesia and hyperalgesia from CRF receptor modulation in the central nervous system of Fischer and Lewis rats. *Pain*. 2006;121(3):241–260.
11. Nijsen M, Ongenae N, Meulemans A, Coulie B. Divergent role for CRF1 and CRF2 receptors in the modulation of visceral pain. *Neurogastroenterol Motil*. 2005;17(3):423–432.
12. Boorse GC, Denver RJ. Widespread tissue distribution and diverse functions of corticotropin-releasing factor and related peptides. *Gen Comp Endocrinol*. 2006;146(1):9–18.
13. Behan DP, Grigoriadis DE, Lovenberg T, et al. Neurobiology of corticotropin releasing factor (CRF) receptors and CRF-binding protein: implications for the treatment of CNS disorders. *Mol Psychiatry*. 1996;1(4):265–277.
14. Tache Y, Larauche M, Yuan PQ, Million M. Brain and gut CRF signaling: biological actions and role in the gastrointestinal tract. *Curr Mol Pharmacol*. Epub 2017 Feb 23.
15. Mousa SA, Bopaiah CP, Richter JF, Yamdeu RS, Schafer M. Inhibition of inflammatory pain by CRF at peripheral, spinal and supraspinal sites: involvement of areas coexpressing CRF receptors and opioid peptides. *Neuropsychopharmacology*. 2007;32(12):2530–2542.
16. Larson AA, Nunez MG, Kissel CL, Kovacs KJ. Intrathecal urocortin I in the spinal cord as a murine model of stress hormone-induced musculoskeletal and tactile hyperalgesia. *Eur J Neurosci*. 2015;42(10):2772–2782.
17. Bakshi VP, Smith-Roe S, Newman SM, Grigoriadis DE, Kalin NH. Reduction of stress-induced behavior by antagonism of corticotropin-releasing hormone 2 (CRH2) receptors in lateral septum or CRH1 receptors in amygdala. *J Neurosci*. 2002;22(7):2926–2935.
18. Fowler M, Clifford JL, Garza TH, et al. A rat model of full thickness thermal injury characterized by thermal hyperalgesia, mechanical allodynia, pronociceptive peptide release and tramadol analgesia. *Burns*. 2014;40(4):759–771.
19. Green DP, Ruparel S, Roman L, Henry MA, Hargreaves KM. Role of endogenous TRPV1 agonists in a postburn pain model of partial-thickness injury. *Pain*. 2013;154(11):2512–2520.
20. Nyland JE, McLean SA, Averitt DL. Prior stress exposure increases pain behaviors in a rat model of full thickness thermal injury. *Burns*. 2015;41(8):1796–1804.
21. Khasar SG, Dina OA, Green PG, Levine JD. Sound stress-induced long-term enhancement of mechanical hyperalgesia in rats is maintained by sympathoadrenal catecholamines. *J Pain*. 2009;10(10):1073–1077.
22. Cheppudira BP. Characterization of hind paw licking and lifting to noxious radiant heat in the rat with and without chronic inflammation. *J Neurosci Methods*. 2006;155(1):122–125.
23. Dominguez E, Rivat C, Pommier B, Mauborgne A, Pohl M. JAK/STAT3 pathway is activated in spinal cord microglia after peripheral nerve injury and contributes to neuropathic pain development in rat. *J Neurochem*. 2008;107(1):50–60.
24. Cheppudira BP, Garza TH, Petz LN, Clifford JL, Fowler M. Anti-hyperalgesic effects of AG490, a Janus kinase inhibitor, in a rat model of inflammatory pain. *Biomed Rep*. 2015;3(5):703–706.
25. Vahl TP, Ulrich-Lai YM, Ostrander MM, et al. Comparative analysis of ACTH and corticosterone sampling methods in rats. *Am J Physiol Endocrinol Metab*. 2005;289(5):E823–E828.
26. LaBerge J, Malley SE, Girard B, Corrow K, Vizzard MA. Postnatal expression of corticotropin releasing factor (CRF) in rat urinary bladder. *Auton Neurosci*. 2008;141(1–2):83–93.
27. LaBerge J, Malley SE, Zvarova K, Vizzard MA. Expression of corticotropin-releasing factor and CRF receptors in micturition pathways after cyclophosphamide-induced cystitis. *Am J Physiol Regul Integr Comp Physiol*. 2006;291(3):R692–R703.
28. Zhang C, Wang B, Wang M. GM-CSF and IL-2 as adjuvant enhance the immune effect of protein vaccine against foot-and-mouth disease. *Virol J*. 2011;8:7.

29. Sosanya NM, Huang PP, Cacheaux LP, et al. Degradation of high affinity HuD targets releases Kv1.1 mRNA from miR-129 repression by mTORC1. *J Cell Biol*. 2013;202(1):53–69.
30. Devall AJ, Liu ZW, Lovick TA. Hyperalgesia in the setting of anxiety: sex differences and effects of the oestrous cycle in Wistar rats. *Psychoneuroendocrinology*. 2009;34(4):587–596.
31. Khasar SG, Green PG, Levine JD. Repeated sound stress enhances inflammatory pain in the rat. *Pain*. 2005;116(1–2):79–86.
32. Dina OA, Levine JD, Green PG. Enhanced cytokine-induced mechanical hyperalgesia in skeletal muscle produced by a novel mechanism in rats exposed to unpredictable sound stress. *Eur J Pain*. 2011;15(8):796–800.
33. Itoga CA, Roltsch Hellard EA, Whitaker AM, et al. Traumatic stress promotes hyperalgesia via corticotropin-releasing factor-1 receptor (CRFR1) signaling in central amygdala. *Neuropsychopharmacology*. 2016;41(10):2463–2472.
34. Abdelhamid RE, Kovacs KJ, Pasley JD, Nunez MG, Larson AA. Forced swim-induced musculoskeletal hyperalgesia is mediated by CRF2 receptors but not by TRPV1 receptors. *Neuropharmacology*. 2013;72:29–37.
35. Tan W, Zhou W, Luo HS, Liang CB, Xia H. The inhibitory effect of melatonin on colonic motility disorders induced by water avoidance stress in rats. *Eur Rev Med Pharmacol Sci*. 2013;17(22):3060–3067.
36. Forbes SC, Cox HM. Peptide YY, neuropeptide Y and corticotrophin-releasing factor modulate gastrointestinal motility and food intake during acute stress. *Neurogastroenterol Motil*. 2014;26(11):1605–1614.
37. Tache Y, Martinez V, Wang L, Million M. CRF1 receptor signaling pathways are involved in stress-related alterations of colonic function and viscerosensitivity: implications for irritable bowel syndrome. *Br J Pharmacol*. 2004;141(8):1321–1330.
38. Tache Y. Corticotropin releasing factor receptor antagonists: potential future therapy in gastroenterology? *Gut*. 2004;53(7):919–921.
39. Bergamini MR, Kabadayan F, Bernardi MM, et al. Stress and its role in the dentin hypersensitivity in rats. *Arch Oral Biol*. 2016;73:151–160.