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Original Article

An Experimental Study on Spinal Cord μ-Opioid and α2-Adrenergic Receptors mRNA Expression Following Stress-Induced Hyperalgesia in Male Rats

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Abstract

Background: Intense stress can change pain perception and induce hyperalgesia; a phenomenon called stress-induced hyperalgesia (SIH). However, the neurobiological mechanism of this effect remains unclear. The present study aimed to investigate the effect of the spinal cord μ-opioid receptors (MOR) and α2-adrenergic receptors (α2-AR) on pain sensation in rats with SIH.

Methods: Eighteen Sprague-Dawley male rats, weighing 200-250 g, were randomly divided into two groups (n=9 per group), namely the control and stress group. The stress group was evoked by random 1-hour daily foot-shock stress (0.8 mA for 10 seconds, 1 minute apart) for 3 weeks using a communication box. The tail-flick and formalin tests were performed in both groups on day 22. The real-time RT-PCR technique was used to observe MOR and α2-AR mRNA levels at the L4-L5 lumbar spinal cord. Statistical analysis was performed using the GraphPad Prism 5 software (San Diego, CA, USA). Student's *t* test was applied for comparisons between the groups. $P<0.05$ was considered statistically significant.

Results: There was a significant ($P=0.0014$) decrease in tail-flick latency in the stress group compared to the control group. Nociceptive behavioral responses to formalin-induced pain in the stress group were significantly increased in the acute ($P=0.007$) and chronic ($P=0.001$) phases of the formalin test compared to the control group. A significant reduction was also observed in MOR mRNA level of the stress group compared to the control group ($P=0.003$). There was no significant difference in α2-AR mRNA level between the stress and control group.

Conclusion: The results indicate that chronic stress can affect nociception and lead to hyperalgesia. The data suggest that decreased expression of spinal cord MOR causes hyperalgesia.

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Keywords ● Stress ● Hyperalgesia ● Spinal cord ● Receptors, opioid, mu ● Adrenergic alpha-2 receptor antagonists

What's Known

- It is known that chronic stress can change nociception and lead to stress-induced hyperalgesia (SIH) and stress-induced analgesia (SIA).
- Unlike SIH, the role of neuronal circuits in SIA has been proven in many studies.

What's New

- The effect of chronic stress on the spinal cord μ-opioid receptors leading to hyperalgesia is demonstrated.

Introduction

Intense stress and fear are the main factors that suppress pain, a phenomenon called stress-induced analgesia (SIA).[1]

Stress can also drastically exacerbate the sensitivity to pain, which is called stress-induced hyperalgesia (SIH).[2] SIA is controlled by brainstem pain modulatory systems,[3] whereas the neural circuitry as the main cause of SIH remains unknown. As confirmed by previous studies, SIA is mediated and modulated by a number of neurotransmitter and neuromodulator systems such as opioids, gamma-aminobutyric acid (GABA), glutamate, monoamines, endocannabinoids, and hypothalamic-pituitary-adrenal (HPA) axis hormones.[4]

A large number of studies on human and animal models have confirmed that the endogenous opioid system plays an important role in the modulation of pain and stress.[5-7] The precursor of the adrenocorticotropic hormone (ACTH) and the opioid peptide β-endorphin is the same at the neuroendocrine level, which responds to similar regulatory mechanisms and is concurrently secreted by the adenohypophysis in response to stress or pain.[8] In the peripheral nervous system, the existence of pain leads to the production of opioid peptides by the dorsal root ganglia neurons and immune cells, which inhibit calcium and sodium ion channels and block firing of nociceptors.[9] Opioid receptors and their corresponding agonists are the main factors in pain prevention and modulation. MOR is one of the most important opioid receptors which plays a key role in analgesia and pain modulation.[10] On the other hand, adrenergic neurotransmission is the crucial aspect in the modulation of pain and stress response pathways.[11] Some studies have confirmed that hyperalgesia is often associated with improved adrenergic sensitivity of primary afferent neurons.[12] One potential mechanism for the increased adrenergic sensitivity of primary afferent neurons is the upregulation of α2-AR.[8]

The present study aimed to assess changes in the spinal cord (L4-L5 segments) MOR and α2-AR gene expression in a rat model of SIH. Supporting arguments to instigate this research were: (i) the role of MOR and α2-AR in descending pain modulatory system and distribution of these receptors in all parts of the spinal cord, (ii) lower limbs pain afferents enter the L4-L5 segments of the spinal cord, and (iii) lack of clarity about SIH.

Materials and Methods

Animals

In the present experimental study, 18 Sprague-Dawley male rats (200-250 g) were randomly divided into two groups, namely the control (n=9) and stress (n=9) group. The stress group was subjected to electric foot-shock stress, 1 hour daily for 3 weeks. The control group was placed in a communication box, 1 hour daily for 3 weeks, without being subjected to electric foot-shock stress. After 3 weeks, behavioral and molecular tests were performed. All rats were maintained at room temperature (25±2 ºC) under standard 12:12 hour light:dark cycle with lights on at 7:00 AM. Food and water were provided except for the duration of the tests.[13]

Protocol for Stress Induction

A communication box was used as a stress stimulus device. This device was divided into nine compartments (16×16×50 cm) using transparent plastic sheets. The box was connected to a stimulator capable of generating an electrical current up to 10 mA (Stimulator 10MA ST5500, Borj Sanat Aazma, Iran). In each session, nine rats (the stress group) were exposed to physical stress by electric foot-shock (1 hour daily, 1 mA at 1 Hz for 10 seconds, 1-minute apart) through stainless steel grids.[14] All rats were acclimatized to the laboratory condition for 30 minutes before being placed in the communication box. The stress group was subjected to a daily session (random times) of foot-shock stress for 21 consecutive days (chronic stress experiment). The control group was placed in the communication box (1 hour daily) for 21 days without inducing stress. All phases of the experiment were conducted in full compliance with the NIH guideline (NIH publication, number: 85-23, revision: 1996). The study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (code: 9371-7351).

Tail-Flick Test

The tail-flick test is based on the withdrawal of the tail (tail-flick) in reaction to noxious cutaneous thermal stimulation induced by an automated tail-flick analgesia meter unit (TSE-Systems, Bad Homburg, Germany; Cat. 7360). The test was conducted based on the method by Stephan.[15] One-third end of the rat's tail was exposed to an infrared heat source with 50 W power. Three sequential measurements were conducted to obtain the mean tail-flick response latency; each time a different section of the tail skin was stimulated. An automatic cutoff circuit was set to 14 seconds in case of accidental beam triggering (due to mismatch with the intended plantar area of the tail) or if the animal did not respond.

Formalin Test

Formalin-related behaviors were observed through a clear plastic box (40×30×30 cm) which

was located above a mirror tilted at 45-degree angle to permit a clear view of the four paws. Prior to the test, rats were acclimatized to the enclosure for 30 minutes. Then, the animals received a 50 µL subcutaneous injection of diluted 2% formaldehyde (product code: 104003, Formaldehyde 37%, Germany) into the plantar surface of the contralateral paw to induce chronic injury. Immediately after the injection, rats were placed in the plastic box and their behavior was monitored for 60 minutes. The intraplantar injection of formalin creates a biphasic nociceptive response. The two distinguished phases of spontaneous pain behavior that occurred following formalin injection were measured. Generally, the formalin test consists of two phases: the acute phase (phase 1): from 0 to 9 minutes after injection and the chronic phase (phase 2): from 21 to 60 minutes after injection. The acute phase score was used to measure rapid pain and the chronic phase score was used to measure slow pain.[16] A nociceptive mean score was set at each 5-minute interval by evaluating the amount of time spent in each of the four behavioral stages (or categories):

Stage 0: The injected paw is similar to the contralateral paw of the control animal.

Stage 1: The injected paw has slight or no weight placed on it.

Stage 2: The injected paw is raised and does not have contact with any surface.

Stage 3: The injected paw is shaken, nibbled, or licked.

The nociceptive score, ranging from 0 to 3, was computed by multiplying the elapsed time in each behavioral stage with the defined weight category; then, the values were summed and divided by the entire time for each 5-minute time segment.[17]

Tissue Collection, RNA Isolation, and cDNA Synthesize

After 3 weeks of exposure to electric foot-shock stress, the stress and control rats were sacrificed. The total RNA was extracted from 80 mg of lumbar spinal cord tissue using Biozol (catalog number: BSC51M1), according to the prescribed protocol of total RNA extraction. The integrity of extracted RNA was confirmed on 1% agarose gel and its concentration was measured by NanoDrop (Thermo Fisher Scientific, USA). Then, 1 µg of the total RNA was treated with DNase I to remove any traces of contaminating DNA, using the DNA-free kit (Ambion, TX, USA), and subsequently reversed transcribed into cDNA according to the instructions of RevertAid First Strand cDNA Synthesis kit.[18]

Primers

Specific primers were designed using AlleleID7.81 software (PREMIER Biosoft, Palo Alto, CA, USA). MOR 5′GCG GTC TGC CAC CCT GTC3′ (sense) and 5′CAC GAA GGC GAA GAG GAA CAC3′ (anti-sense); α2-AR 5′GTG TGC TTG TTT CTG TCT TG3′ (sense) and 5′TAT CGG GTA GGT TTC TTC CA3′ (anti-sense). For standardization, β-actin was used as a reference gene and the sequence of its primers was: 5′ CGA CAG CAG TTG GTT GGA GC3′ (sense) and 5′ GGT CTC AAG TCA GTG TAC AG3′ (anti-sense) (table 1).

Real-Time Reverse Transcriptase PCR

The reverse transcription (RT) was performed in a 100 ml total reaction volume comprising dNTPs, RT buffer, RNase-free water, MultiScribe reverse transcriptase, and 10 mg of DNase-treated (Thermo Scientific, USA) total RNA. The RT reaction was done at 25 ºC for 10 minutes, 37 ºC for 120 minutes, and 95 ºC for 5 minutes in the Mastercycler Gradient Eppendorf (Brinkmann Instrument Inc, NY, USA). Quantitative real-time PCR (QRT-PCR) was done in the Applied Biosystems 7500 real-time PCR system using SYBR Green dye for quantitative detection of the expressed target gene.[19] Each reaction mixture contained 25 µl total volume and included SYBR Green PCR master mix 12.5 µl, the reverse transcribed diluted cDNA with 1:5 ratio (as template), specific forward and reverse primers for opioid receptors (as mentioned above), and total volume equal to 25 µl with nuclease-free water for PCR. The amplification reaction was performed with the following cycling conditions: 15 minutes for initial heat activation at 95 °C, followed by 40 cycles of

Table 1: Specific primer sequences and their product size used in RT-PCR for MOR, α2-AR, and β-actin genes

Assay	Primer	Sequence	Annealing temperature (°C)	Product size (bp)
RT-PCR	MOR-F	5´- GGTGGTCGTGGCTGTATT -3´	60	170
	MOR-R	5´- AAGGCGTAAAGAACTGGAT -3´		
	α2-AR-F	5´- GTGTGCTTGTTTCTGTCTTG -3´	60	140
	α2-AR-R	5´- TATCGGGTAGGTTTCTTCCA -3´		
	β-actin-F	5´- CCACACCCGCCACCAGTTCG -3´	60	148
	β-actin-R	5´- CTAGGGCGGCCCACGATGGA -3´		

F: Forward; R: Reverse

denaturation at 95 ºC for 25 seconds, annealing at 60 ºC for 25 seconds, and extension at 72 ºC for 20 seconds. The mRNA relative expression levels, measured by fluorescence signal intensity, were gathered at the end of each reaction. Curves were analyzed by the Prism software. The relative expression level of each mRNA was normalized to the endogenous β-actin reference gene. Expression ratio was calculated using the Pfaffl method (2-ΔΔCT).[20] All the PCRs were performed at least three times in separate runs and expression was presented as a fold change relative to its respective control group.

Experimental Protocol

After an acclimation period of a week, the stress group was subjected to a chronic stress protocol for 3 weeks. The control group was placed in the communication box (a random 1 hour daily) without being exposed to electrical shock. On day 22, somatic pain sensitivity was assessed using the thermal nociceptive tail-flick reflex and formalin test. The animals were sacrificed using CO_2 and their spinal cord was dissected to analyze changes in the desired gene expression level by real-time RT-PCR.

Statistical Analysis

Statistical analysis and graphic design were performed using GraphPad Prism 5 software (San Diego, CA, USA). Student's *t* test was performed to compare unpaired groups for a single dependent variable. In all statistical comparisons, P<0.05 was considered as a significant difference.

Results

Somatic nociceptive responses were investigated following chronic (3 weeks) electric foot-shock stress. Tail-flick latency was measured in both the control and stress groups on day 22. There was a significant decrease in tail-flick latency in the stress group compared to the control group (P=0.0014) (figure 1). Formalin was injected in the contralateral hind paw to evaluate descending pain modulation in reaction to tonic pain at various intervals following exposure to chronic foot-shock stress. Nociceptive behavioral responses to formalin-induced pain in the stress group were significantly increased in the acute (P=0.0074) and chronic (P=0.0011) phases of the formalin test compared to the control group (figure 2).

Changes in MOR and α2-AR mRNA following chronic stress were also investigated. The real-time RT-PCR technique was used to identify MOR and α2-AR mRNA levels at the L5 lumbar spinal cord of the rats. The mRNA levels of μ-opioid and α2-AR receptors in the lumbar

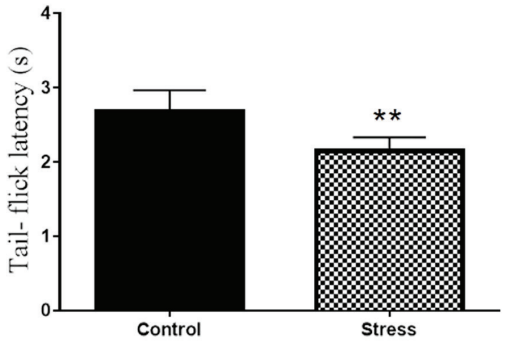

Figure 1: After 3 weeks exposure to electric foot-shock stress, tail-flick latency was decreased in the stress group compared to the control group (n=9 per group). The data (in seconds) correspond to the latency before tail withdrawal and expressed as mean±SEM. **Difference between the control and stress group (P=0.0014)

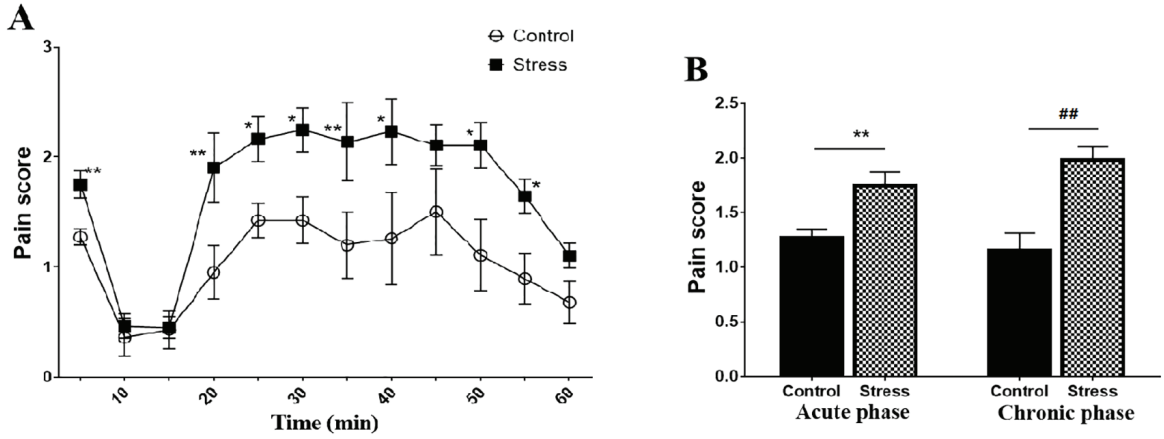

Figure 2: Nociceptive indices are shown as: (A) time course and (B) mean score of the acute (0-9 min) and the chronic (21-60 min) phases after formalin injection in the contralateral hind paw of the control and stress group (n=9 per group). Nociceptive mean scores were determined at each 5-minute interval by measuring the amount of time spent in each of the four behavioral categories (see section "Formalin Test" for details). Data are represented as mean±SEM. **P=0.0074 in the acute phase (0-9 minutes); ##P=0.0011 in the chronic phase (21-60 minutes).

spinal cord of the control and stress group are shown in figure 3. The results showed that the stress group had a significantly lower level of μ-opioid receptor mRNA compared to the control group (P=0.0038). Additionally, no considerable change was observed in the mRNA levels of α2-AR at the L5 lumbar spinal cord of the stress group compared to the control group.

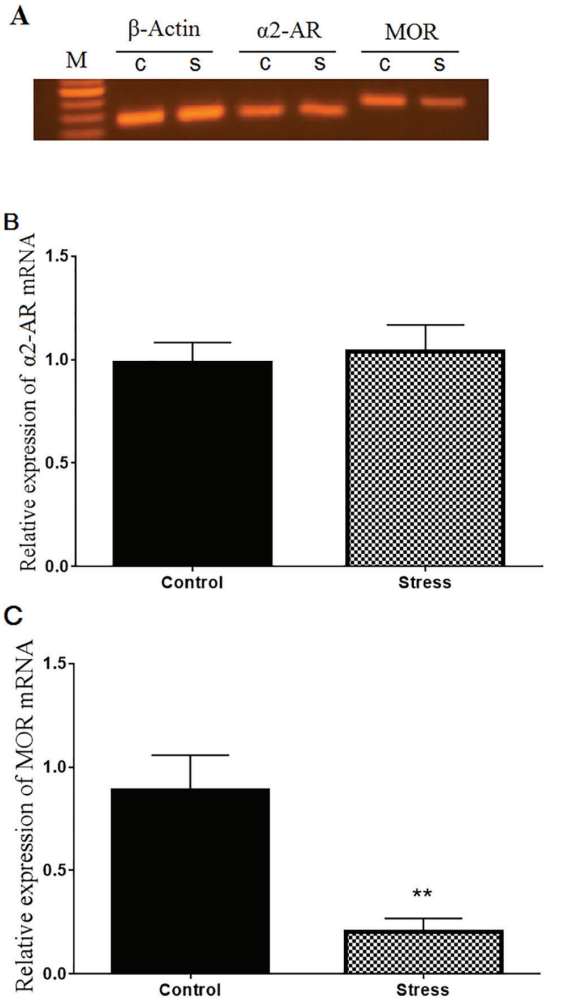

Figure 3: Representation of MOR and α2-AR mRNA expression of the spinal cord (L4-L5) in the stress and control group (n=9 per group). Values are expressed as mean±SEM. (A) GelRed stained agarose gel electrophoresis of RT-PCR product of the control and stress group. One microgram of RNA was reverse transcribed and cDNA was amplified for 40 cycles. M: Molecular weight marker, C: The control group in 3 different genes, S: The stress group in 3 different genes. (B) Comparison of α2-AR mRNA expression level in the lumbar spinal cord after chronic foot-shock stress between the control and stress group. (C) Dynamic changes in MOR mRNA expression level in the lumbar spinal cord after chronic foot-shock stress in the stress group compared to the control group (**P=0.0038).

Discussion

In the present study, it was demonstrated that a long-term electric foot-shock stress leads to hyperalgesia. It was also found that chronic stress resulted in down-regulation of MOR mRNA in the spinal cord. There are several different methods to induce SIA and SIH (e.g. water deprivation, tail clamping, fasting, forced-swim test in cold water; electric foot-shock stress, thermal and sound stimulation, converting the circadian rhythm, tilting cages). Each of these can influence biological activities in different ways. To induce stress, we applied the electric foot-shock stress method by using a communication box. The results showed that 3 weeks of foot-shock stress influenced nociception and resulted in hyperalgesia.

On day 22, there was a significant decrease in tail-flick latency in the stress group compared to the control group. Also, nociceptive behavioral responses to formalin-induced pain in the stress group meaningfully increased in the acute and chronic phases of the formalin test compared to the control group. These findings are in line with previous studies. To date, factors that could determine the difference between SIA and SIH are unknown. However, stressor intensity or diversity can interact with the stimulation level to identify whether the net impact augments or suppresses the pain.[21, 22] The results of the formalin test indicated that chronic stress can influence the acute and chronic phases. These phases quantify fast and slow pain perceptions since they have their own separate afferent pathways. Hence, as shown by our results, chronic stress may affect both the slow and fast pain afferents.

It is well established that the two types of descending pathways, opioid and non-opioid pathways, play a key role in spinal pain modulation.[23] We investigated the role of MOR and α2-AR gene expressions as part of the opioid and non-opioid pathways, respectively. Real-time PCR revealed a significant decrease in the spinal cord MOR mRNA of the stress group compared to the control group. Moreover, there was no significant difference between the stress and control group in terms of α2-AR gene expression at mRNA level.

Adrenergic neurotransmitter has an essential role in the regulation of pain and stress response pathways. Some studies have demonstrated that hyperalgesia is often attributed to increased adrenergic sensitivity of primary afferent neurons. Sensory nerves in animals can be sensitized by conditions such as sympathetic nerve excitation or exogenous injection of the sympathetic adrenergic neurotransmitter, norepinephrine, following nerve injury or when inflammatory mediators are present.[24, 25] Possible mechanisms for the enhanced primary afferent adrenergic sensitivity

include upregulation of α2-AR.[23] Numerous levels of the stress pathway are fundamentally regulated by α2-AR. All the above-mentioned experiments revealed a balance between pain facilitatory/hyperalgesic and pain inhibitory/analgesic mechanisms triggered by stress that was changed into analgesia by α2-receptor activity. Considering the location of different types of adrenergic receptors (α2A and α2C) in the dorsal horn of the spinal cord,[11] studies with α2A-knockout mice have revealed that α2A is essential for spinal analgesia with α2-agonists and the α2C adrenergic receptor interacts with the opioid system in the spinal cord.[26, 27] The absence of SIA after treatment with different α2-antagonists and the α2A-knockout mice showed a physiological role for the spinal α2-AR. This is in line with pharmacological studies using α2-agonists which confirmed that descending pain inhibitory projections were mediated by α2-AR.[11, 28, 29] Furthermore, SIH was more intensive in α2A-knockout mice and in animals treated with α2-AR antagonists due to mediated pain afferent. This reflects the crucial role of α2-adrenergic receptors in attenuating pain signaling pathway. The fact that hyperalgesia or analgesia could be induced by both a psychological stressor and a physical stressor, indicated that it may include the same pathway stimulated by different types of stressors. In spite of the differences between both stressors, changes in pain perception could be affected by α2-AR.[12]

It was found that changes in α2-AR gene expression did not play any role in the SIH condition. Probably some other mechanisms (e.g. receptor desensitization) were involved in this process. Moreover, it is well documented that morphine and endogenous opioid ligands exert their different functions in both the central and peripheral nervous systems by MOR activation. In addition, the analgesic function of morphine can be affected by the level of MOR expression.[10, 30] Our previous research indicated that enhanced expression of spinal MOR at protein level resulted in hyperalgesia reduction during the chronic inflammatory phase of adjuvant-induced arthritis.[31] Additionally, several studies have demonstrated that chronic stress can enhance MOR expression in different areas of CNS.[32-34] On the contrary, a large amount of information has indicated that MOR expression is decreased in the spinal cord and dorsal root ganglion following nerve damage.[35] Such reduction in MOR expression somewhat explains why the analgesic effect of opioids is attenuated during neuropathic pain. Nevertheless, the molecular mechanism causing these dynamic changes in MOR expression following nerve damage has not been well studied. It seems that alteration in MOR expression can be regulated by changes in DNA methylation patterns.[36, 37] Therefore, chronic stress may induce hyperalgesia not only through the spinal cord MOR gene methylation but also via a decrease at its mRNA level.

Due to the presence of various stressors in human life and their important role in changing biological processes, it is recommended to examine the role of these stressors in terms of pain. To study the effect of stress on pain sensation, one should evaluate the spinal cord pain modulatory system. These neuronal pathways are very complex and have many neurotransmitters. Additionally, there are many different genes involved in the regulation of neuronal function. To examine all these neurotransmitters and genes, more funding and laboratory equipment are required. For instance, microarray technology can be used to examine a large number of genes. Considering the importance of pain and pain relief in clinical processes, there is a need for more extensive studies in this field.

Conclusion

Three weeks of electric foot-shock stress, using a communication box, in adult male rats induced hyperalgesia due to reduced expression of MOR. Chronic stress seems to affect spinal cord opioidergic pathways and cause changes in nociception. In addition, other spinal pathways may be involved in the induction of this hyperalgesia. Because of the importance of pain and pain relief in clinical interventions, chronic stress appears to dramatically change the process of pain sensation.

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Conflict of Interest: None declared.

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