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Co-Localization of p-CREB and p-NR1 in Spinothalamic Neurons in a Chronic Muscle Pain Model

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Abstract: Activation of the cAMP pathway is an important mediator of chronic muscle pain. This study examined phosphorylation of the transcription factor cAMP-response-element-binding protein (p-CREB) and the NR1 subunit of the NMDA receptor (p-NR1) in the spinal cord. Bilateral mechanical hyperalgesia of the paw was induced by administering two injections of acidic saline, 5 d apart, into the gastrocnemius muscle of male Sprague Dawley rats. The proportion of spinothalamic neurons that expressed p-NR1 or p-CREB did not change in the dorsal horn 24 hours after the second intramuscular acid injection compared with animals that received pH 7.2 injections. This lack of change in spinothalamic neurons in the dorsal horn may be due to increases in individual spinothalamic neurons or increases in non-spinothalamic neurons. There was an increase in the proportion of spinothalamic neurons expressing p-NR1 in lamina X. These findings suggest that there are region-

specific changes in spinothalamic neurons that express p-NR1 and lamina X may play an important role in the modulation of chronic muscle pain.

Keywords: cAMP pathway, protein kinase A, phosphorylation, nociception

Introduction

Activation of the cAMP pathway has an integral role in nociceptive processing. Spinal activation of the cAMP pathway produces mechanical hyperalgesia and increases the response of spinothalamic tract neurons to noxious but not innocuous mechanical stimuli [12, 16, 17]. Mice lacking adenylate cyclases 1 and 8 have no changes in acute pain but have decreases in behavioral responses after administration of formalin, complete Freund's adjuvant (CFA), and intramuscular carrageenan [20, 21]. Furthermore, blocking adenylate cyclase or protein kinase A (PKA) prevents the mechanical hyperalgesia and allodynia produced by intradermal, intramuscular, or intraarticular injection of capsaicin, and repeated intramuscular acidic saline injections [9, 17, 19].

The catalytic subunit of PKA translocates to the nucleus and phosphorylates cAMP-response-element-binding protein (CREB) at serine-133, which is necessary for gene transcription. An increase in phosphorylated-CREB (p-CREB) occurs in a number of pain models such as carrageenan paw inflammation [13], intradermal capsaicin [22], subcutaneous formalin [10, 21], nerve growth factor [7], neuropathic pain [14], and repeated intramuscular acid injections [9]. Furthermore, p-CREB corresponds to the time frame of hyperalgesia in neuropathic and inflammatory pain [10, 14], and there is an upregulation of p-CREB in spinothalamic neurons after spinal cord injury [4]. Thus, phosphorylation of CREB is involved in central nervous system changes associated with hyperalgesia.

PKA also phosphorylates the NMDA receptor at serine-897 of the NR1 subunit (p-NR1). Protein kinases functionally modulate the NMDA receptor through phosphorylation. Specifically, phosphorylation by intracellular messengers promotes receptor trafficking and alter cell kinetics. Similar to p-CREB, there are increases in p-NR1 in several animal models of nociception: intradermal capsaicin [24, 25], neuropathic [6], and carrageenan paw inflammation [3]. Following

intradermal capsaicin, p-NR1 increases in nociceptive specific neurons and this increase is prevented by blocking PKA [24, 25]. Following excitotoxic spinal cord injury, excessive grooming is associated with phosphorylation of NR1 [3]. Thus, phosphorylation of the NMDA receptor by PKA is an important mediator of pain transmission.

We have previously shown that increases in p-CREB and mechanical hyperalgesia in a chronic muscle-induced hyperalgesia model are dependent on activation of the cAMP pathway [9]. Specifically, there is a time-dependent increase in the density of immunoreactivity for p-CREB. This increase could represent an increase in the amount of p-CREB or number of cells expressing p-CREB. The purpose of this study is to determine if there is an increase in the number of spinothalamic neurons that express p-CREB or p-NR1 following chronic muscle-induced hyperalgesia. We hypothesize there will be an increase in the number of spinothalamic neurons that express p-CREB and p-NR1.

Methods

All methods were approved by the University of Iowa's Animal Care and Use Committee and followed the policies issued by the International Association for the Study of Pain and National Institutes of Health on the use of laboratory animals. Male Sprague Dawley rats were used in all of the experiments (250–350 g; Harlan, St. Louis, MO).

Chronic Muscle-Induced Hyperalgesia Model

Two injections of pH 4.0 sterile saline (100 µl) were administered five days apart in the left gastrocnemius muscle while the rats were anesthetized with vaporized halothane (2–4%) [18]. This model produces long-lasting bilateral mechanical hyperalgesia with minimal to no muscle tissue damage or motor deficits [18]. In some animals, two intramuscular injections of pH 7.2 (100 µl) sterile saline were used for controls.

Fluorogold Injections into Thalamus

Fluorogold injections into the thalamus were performed to co-localize spinothalamic neurons with either p-CREB or p-NR1. Rats were anesthetized with Nembutal (50 mg/kg, i.p.) and placed in a stereotactic frame for thalamic injections. Four injections of 2% fluorogold (1 μ l total) were given on each side (ipsilaterally and contralaterally) into the thalamus using a Hamilton microsyringe based on coordinates from *The Rat Brain in Stereotactic Coordinates* [15]. Thalamus injections were verified for accurate placement by sectioning the rat's thalamus using a cryostat and mapping the injection sites.

Co-localization of Spinothalamic Neurons with p-CREB or p-NR1

Rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused through the left ventricle with 100 ml of heparinized saline followed by 1 liter of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, 4°C. The L5 segment was removed and placed in 30% sucrose solution. Tissue was cut, using a cryostat, into 40 μ m sections and placed in PB. Sections were alternately placed in the p-NR1 or p-CREB vial to ensure that profiles were not counted more than once for each antibody. These sections were exposed to a number of solutions in a step-wise procedure that included 0.5% H₂O₂, 1% NaBorohydride, solution A (Avidin), solution B (Biotin) and 3% normal goat serum. Between each step, tissue was rinsed in phosphate buffered saline (PBS). Next, sections were incubated overnight in primary antibody in 1% normal goat serum/PBS 0.75% Triton X 100 at room temperature as follows: anti-p-CREB (1:2000; goat anti-rabbit; Upstate Biotechnology) or anti-p-NR1 (1:10,000; goat anti-rabbit; Upstate Biotechnology). Preliminary dilution series were conducted to determine appropriate concentrations for p-NR1, p-CREB, and fluorogold antibodies.

Following overnight incubation, sections were washed in PBS and 3% normal goat serum followed by incubation for 1h in the secondary antibody biotinylated-goat anti-rabbit IgG (1:1000). After washing in PBS and 3% normal goat serum, sections were incubated in Avidin-HRP (1% normal goat serum with 0.75 Triton X 100) (1:1000)

for 1h. This step was followed by 6 minutes in nickel-enhanced DAB (Vector Laboratories, Inc., Burlingame, CA). To decrease cross reaction between the two antibodies, tissue was rinsed in PBS six times and once in 1% formalin/PB solution. The day 1 protocol was repeated except the tissue was placed in anti-fluorogold antibody, which was raised in rabbit, (1:200,000, Fluorochrome, Inc) and incubated overnight.

On the third day of labeling, sections were washed in PBS and 3% normal goat serum followed by incubation for 1h in the secondary antibody biotinylated-goat anti-rabbit IgG (1:1000). After washing in PBS and 3% normal goat serum, sections were incubated in Avidin-HRP (1% normal goat serum with 0.75 Triton X 100) (1:1000) for 1h. This step was followed by 6 minutes in DAB (Vector Laboratories, Inc., Burlingame, CA). Sections were washed and mounted on slides, allowed to dry 24–48 hours prior to cleaning in ethanol/water and propar and coverslipped.

Counting Profiles

Spinal cord sections were randomly chosen from each rat. Images were generated using an Olympus BX-51 microscope (Central Electron Microscopy Research Facility, University of Iowa). For co-localization of p-NR1 or p-CREB with spinothalamic neurons, positively double labeled profiles were counted in the following areas: lamina I, deep dorsal horn (laminae III–VI), lamina X, and ventral horn. Approximately 17–25 segments were counted for each animal. The majority of retrogradely labeled spinothalamic neurons were in the deep dorsal horn, approximately 100 cells per animal, compared to the average of 10 cells per animal in the superficial dorsal horn; thus explaining the variability in the co-localization. The proportion of spinothalamic neurons co-localized with p-NR1 or p-CREB were analyzed using a one-way ANOVA. Level of statistical analysis significance was set at $p < 0.05$.

Experimental Design

Fluorogold was injected bilaterally into the rat thalamus. Rats were allowed to recover three days. Following the recovery period,

hyperalgesia was induced by administering two pH 4.0 saline injections into the left gastrocnemius given five days apart. Twenty-four hours following the second injection, immunohistochemistry was performed to identify spinothalamic neurons that contained either p-NR1 or p-CREB. This procedure was repeated using a control group of animals that received two pH 7.2 intramuscular injections. Quantification was determined by counting the number of spinothalamic neurons expressing p-NR1 or p-CREB. Eleven animals were used to complete this study.

Results

Spinothalamic Neurons Express p-CREB

Spinothalamic neurons co-localized with p-CREB (Fig. 1). Animals that received pH 4.0 intramuscular acid injections presented with the following proportion of spinothalamic neurons that expressed p-CREB 24 hours after the second intramuscular acid injection in lamina X (32%), ipsilaterally in lamina I (68%), deep dorsal horn (23%), and ventral horn (19%), and contralaterally in lamina I (60%), deep dorsal horn (24%), and ventral horn (25%) (Fig. 2). Animals that received pH 7.2 intramuscular acid injections presented with the following proportion of spinothalamic neurons co-localized with p-CREB in lamina X (50%), ipsilaterally in lamina I (79%), deep dorsal horn (23%), and ventral horn (15%), and contralaterally in lamina I (86%), deep dorsal horn (26%), and ventral horn (8%) (Fig. 2). The number of spinothalamic neurons that expressed p-CREB remained unchanged in the spinal cord 24 h after the second intramuscular injection of pH 4.0 saline compared with animals that received pH 7.2 intramuscular injections (Fig. 2). Specifically, co-localization of spinothalamic neurons with p-CREB did not significantly differ in lamina X ($p= 0.30$), ipsilaterally in lamina I ($p= 0.42$), deep dorsal horn ($p= 0.99$), and ventral horn ($p= 0.82$), and contralaterally in lamina I ($p= 0.06$), deep dorsal horn ($p= 0.66$), and ventral horn ($p= 0.38$) compared with rats that received pH 7.2 intramuscular injections. Repeated intramuscular acid injections did not induce changes in the co-localization of spinothalamic neurons with p-CREB.

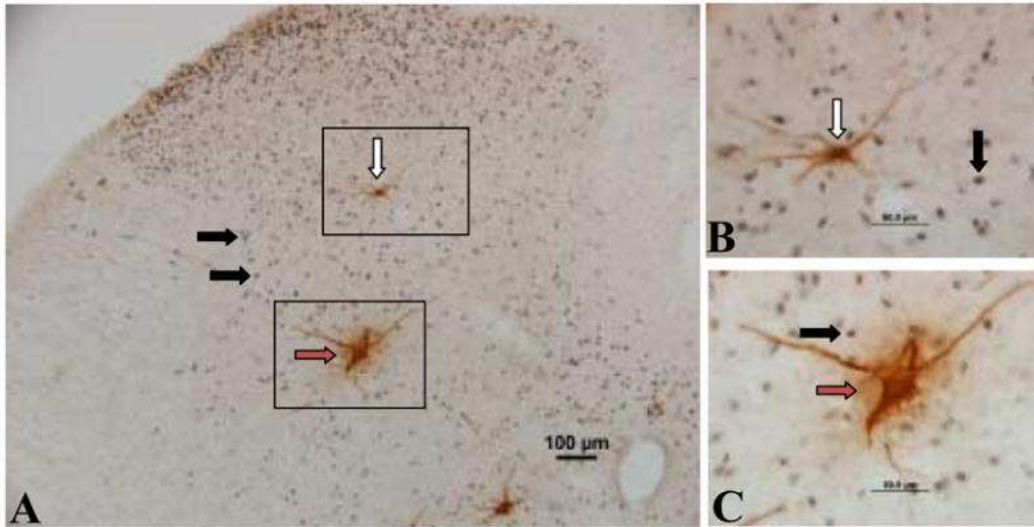


Figure 1 Spinothalamic neuron expressing p-CREB

DAB labeling of spinothalamic tract neurons (brown arrows), nickel-enhanced DAB labeling for p-CREB (black arrows), and co-localization of p-CREB and spinothalamic tract neurons (white arrows). B and C are higher power magnifications of the areas outlined in A.

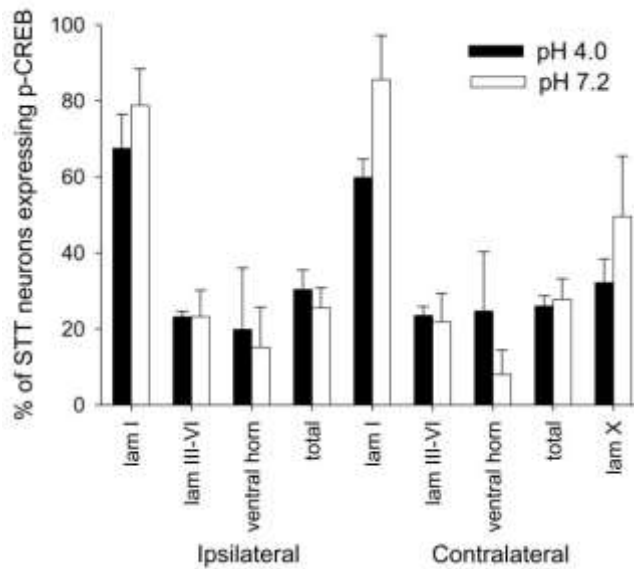


Figure 2 Proportion of spinothalamic neurons expressing p-CREB at 24 hours

The proportion of p-CREB profiles co-localized with spinothalamic neurons is shown for animals that received two intramuscular injections of acidic saline (black bars) and for those that received two intramuscular injections of pH 7.2 saline (open bars). Co-localization was similar between different lamina (lam), and between the ipsilateral

and contralateral sides. There was no significant difference in the proportion of retrogradely labeled spinothalamic neurons with p-CREB in the spinal cord 24 h after the second intramuscular injection of pH 4.0 saline compared with pH 7.2 intramuscular injections. Data are presented as the average with the standard error of the mean.

The pH 4.0 and pH 7.2 injected animals were combined due to the insignificance between pH 4.0 and pH 7.2 injected animals. The number of spinothalamic neurons that expressed p-CREB in pH 4.0 and pH 7.2 injected animals was greater in lamina I ipsilaterally ($p < 0.014$) and contralaterally ($p < 0.016$) compared with the deep dorsal horn, ventral horn, and lamina X.

Spinothalamic Neurons Express p-NR1

Spinothalamic neurons co-localized with p-NR1 (Fig. 3). Animals that received pH 4.0 intramuscular acid injections presented with the following proportion of spinothalamic neurons co-localized with p-NR1 in lamina X (93%), ipsilaterally in lamina I (98%), deep dorsal horn (84%), and ventral horn (73%), and contralaterally in lamina I (79%), deep dorsal horn (82%), and ventral horn (53%) (Fig. 4). Animals that received pH 7.2 intramuscular acid injections presented with the following proportion of spinothalamic neurons co-localized with p-NR1 in lamina X (76%), ipsilaterally in lamina I (96%), deep dorsal horn (81%), and ventral horn (88%), and contralaterally in lamina I (79%), deep dorsal horn (74%), and ventral horn (80%) (Fig. 4). Co-localization of spinothalamic neurons with p-NR1 did not significantly differ ipsilaterally in lamina I ($p = 0.72$), deep dorsal horn ($p = 0.72$), and ventral horn ($p = 0.27$), and contralaterally in lamina I ($p = 0.97$), deep dorsal horn ($p = 0.33$), and ventral horn ($p = 0.23$) compared with rats that received pH 7.2 intramuscular injections. The number of spinothalamic neurons that expressed p-NR1 was significantly greater in lamina X ($p = 0.03$) 24 hours after the second intramuscular injection of pH 4.0 saline compared with rats that received pH 7.2 injections (Fig. 4).

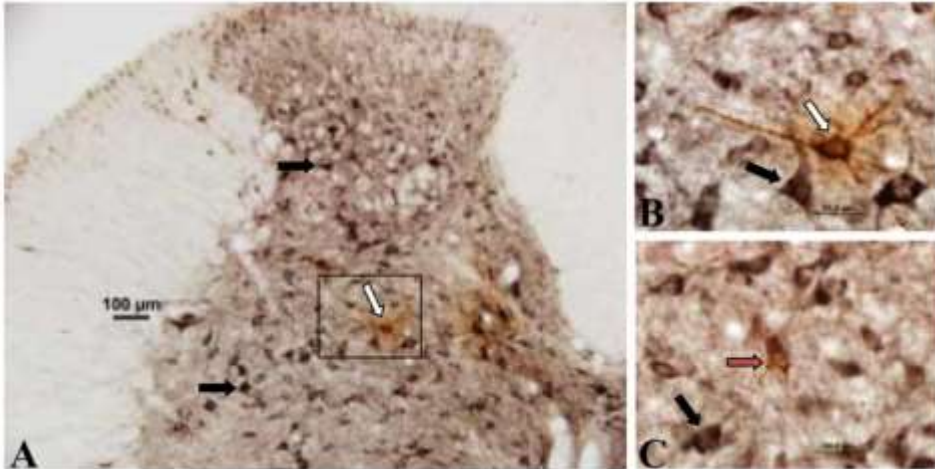


Figure 3 Spinothalamic neuron expressing p-NR1

DAB labeling of spinothalamic tract neuron (brown arrow), nickel-enhanced DAB labeling for p-NR1 (black arrows), and co-localization pNR1 and spinothalamic tract neurons (white arrows). B is a high power magnification of the area outlined in A showing a spinothalamic tract cell labeled for pNR1. C is a high power magnification of a spinothalamic tract cell that is not labeled with pNR1.

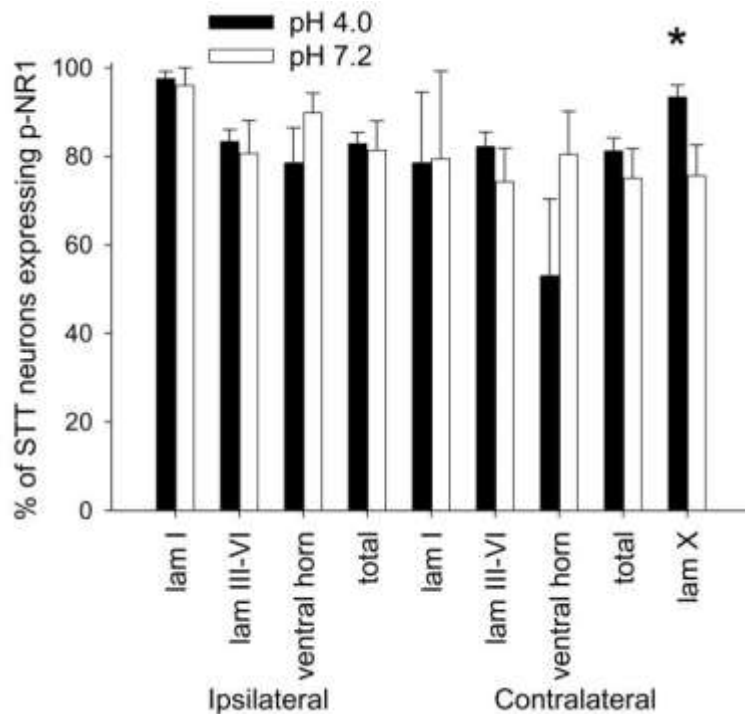


Figure 4 Proportion of spinothalamic neurons expressing p-NR1 at 24 hours

The proportion of p-NR1 profiles co-localized with spinothalamic neurons is shown for animals that received two intramuscular injections of acidic saline (black bars) and for

those that received two intramuscular injections of pH 7.2 saline (open bars). Over 80% of spinothalamic tract neurons co-localized with p-NR1. There was an increase in the number of co-localized neurons in lamina X of the spinal cord, but not in the superficial laminae (lam I), the deeper dorsal horn (lam III–VI), or the ventral horn, for the animals injected with pH 4.0 saline compared with those injected with pH 7.2 saline. Data are presented as the average with the SEM *, $p < 0.05$.

Discussion

Spinothalamic Neurons and p-CREB

This study demonstrates that p-CREB co-localizes with spinothalamic neurons throughout the spinal cord. The proportion of pH 4.0 and pH 7.2 spinothalamic neurons expressing p-CREB was greater in lamina I bilaterally compared with deep dorsal horn, ventral horn and lamina X. Approximately 60% of lamina I spinothalamic neurons were positive for p-CREB compared with ~20% in the deep dorsal horn. Thus, lamina differences may be important for processing nociceptive specific changes involved in gene transcription.

Previously we demonstrated increases in the density of p-CREB in the chronic muscle pain model, which correlated with the mechanical withdrawal threshold [9]. This study shows that there was not an increase in the number of spinothalamic neurons expressing p-CREB, therefore activation of the cAMP pathway likely increases the phosphorylation of CREB in individual spinothalamic neurons or increases the proportion of non- spinothalamic neurons expressing p-CREB. For example, following sciatic nerve transection, the number of glial cells expressing CREB increased in the lumbar spinal cord [8].

In contrast to our findings, Crown and colleagues demonstrated an increase in the proportion of spinothalamic neurons that expressed p-CREB in a spinal cord injury model [4]. All of the spinothalamic neurons in the spinal cord injured rats expressed p-CREB, which correlated with tactile allodynia, compared with 64% of the neurons in the sham rats [4]. These findings indicate that chronic muscle pain and neuropathic pain likely have subtle differences in how activation of the cAMP pathway modulates pain transmission.

Spinothalamic Neurons and p-NR1

This study demonstrates that the majority of spinothalamic neurons express p-NR1. The proportion of spinothalamic neurons that express p-NR1 did not change in the dorsal horn 24 hours after the second intramuscular acid injection compared with animals that received pH 7.2 injections. This lack of change in p-NR1 is unlike previous studies demonstrating increases in the proportion of spinothalamic neurons expressing p-NR1 following an intradermal capsaicin injection [24, 25]. This increase in expression of p-NR1 in neurons was prevented in the superficial laminae (I–III), but not deeper laminae (IV–VII), by pretreatment with a PKA inhibitor [24, 25].

Interestingly, there was an increase in the proportion of spinothalamic neurons expressing p-NR1 in lamina X 24 hours after the second intramuscular acid injection. The increase in lamina X is surprising. Willis and colleagues did not quantify the proportion of spinothalamic neurons expressing p-NR1 in lamina X [24, 25]. However, they did indicate that spinothalamic neurons expressing p-NR1 were present in lamina X [24]. Therefore, the change in lamina X is difficult to compare with the previous literature on spinothalamic neurons expressing p-NR1, which may or may not be specific to muscle pain.

Previous literature supports the role of lamina X in a number of acute and chronic pain conditions including visceral, inflammatory and muscle [1, 5, 11]. For example, following noxious visceral stimulation the expression of immediate early genes in spinal cord neurons increased, which included lamina X [11]. In sheep with footrot, α_2 -adrenoreceptor binding increased in lamina I, II, and X [1]. Furthermore, the proportion of neurokinin-1 neurons expressing Fos was greater following intramuscular injection of mustard oil compared with mustard oil topically applied to the rat hindpaw in laminae V–X [5]. Thus, changes in lamina X demonstrated in the chronic muscle pain model reflect changes expressed in other pain models.

Neurons from lamina X send and receive projections supraspinally. Furthermore, descending projections from supraspinal sites, such as the RVM, release serotonin in the spinal cord. Serotonin

receptors in the spinal cord mediate descending facilitation [2, 23]. Thus, descending facilitatory pathways, which send projections to lamina X, mediate hyperalgesia potentially by releasing serotonin and activating the cAMP pathway. Similarly, in the chronic muscle pain model, descending facilitatory pathways may activate the cAMP pathway and regulate mechanical hyperalgesia.

One of the limitations in this experiment is the high proportion of spinothalamic neurons expressing p-NR1 in the dorsal horn of the control group that received two pH 7.2 intramuscular injections. Thus, increases following repeated intramuscular acid injections would be difficult to obtain significance. This particular protocol may not be sensitive enough to record changes in the expression of p-NR1 in spinothalamic neurons in the dorsal horn.

Conclusion

The expression of p-CREB did not change in spinothalamic tract neurons. Due to the use of density readings to measure the increase in spinal p-CREB in this chronic muscle pain model [9], this lack of change in spinothalamic neurons may be due to increases in individual spinothalamic neurons or increases in non-spinothalamic neurons, such as glial cells, thus suggesting a number of possibilities for future studies.

Similarly, there was no change in the dorsal horn in the proportion of spinothalamic neurons expressing p-NR1. These results are unlike previous literature that demonstrates an increase in the proportion of spinothalamic neurons expressing p-NR1 or p-CREB in an inflammatory cutaneous pain model or neuropathic pain model, respectively [4, 24, 25]. Therefore, this lack of change in p-NR1 and p-CREB in the dorsal horn may be specific to chronic muscle pain.

Surprisingly, there was an increase in the proportion of spinothalamic neurons expressing p-NR1 in lamina X. There is difficulty in comparing this increase with previous literature since lamina X was not measured. Hence, the increase in proportion of spinothalamic neurons expressing p-NR1 in chronic muscle pain is an exciting discovery potentially indicating region-specific changes.

Footnotes

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