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Microinjection of calcitonin in midbrain periaqueductal gray attenuates hyperalgesia in a chronic constriction injury rat model

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ABSTRACT

Objective(s): As heat, pain is one of the most common clinical symptoms. Generally, calcitonin (CT) is prescribed as an analgesic agent for the treatment of pain, especially for the pain caused by osteoporosis or primary and metastatic bone tumor. However, the detailed mechanism remains unknown.

***Materials and Methods*:** In this study, chronic constriction injury (CCI) rat model was created, and hot plate test and von frey filaments test were employed to evaluate thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT), respectively. Immunohistochemistry staining and western blot analyses were used to assess the distribution and expression of calcitonin receptor (CT-R) in the midbrain periaqueductal gray (PAG), which was a pivotal site in the modulatory system for the endogenous pain.

Results: The TWL and MWT before the surgery (19.6±1.19 sec) were significantly longer than that at day 2 (12.5±1.60 sec), and day 14 (7.75±0.89 sec) ($P< 0.01$; $P< 0.01$), respectively. The TWL and MWT at day 14 were significantly increased compared to that at day 7 after microinjection of salmon calcitonin (sCT) with different doses. Interestingly, the expression of CT-R was up-regulated in neuropathic pain. Furthermore, the expression of CT-R was significantly up-regulated and algesia was remarkably relieved when CT was microinjected into PAG.

Conclusion: These results suggested that an increased CT-R might be associated with hyperalgesia in CCI rat, and CT had a potent antinociceptive effect by the up-regulation of CT-R in the PAG. Thus, it might provide a potential approach for the treatment of bone pain.

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Introduction

Chronic neuropathic pain is a serious issue resulting from injury to the central or peripheral nervous system. The pathway from the midbrain periaqueductal gray (PAG) through the ventromedial medulla (VMM) to the dorsal horn constitutes a putative endogenous nociceptive modulatory system (1). Generally, the PAG is regarded as a core of this analgesic system. The PAG receives input signals from several brain regions and in turn projects them into the rostral ventromedial medulla, which relays midbrain signals from spinal nociceptive inputs; i.e., descending modulation of pain (2-5). Recently, it has been confirmed that the PAG participated in the pain modulation of several types of neuropathic pain such as diabetic neuropathy (6, 7).

It has been found that some analgesic drugs or therapy strategies relieved pain partially via affecting the neurons within the PAG (8, 9). Calcitonin (CT), a 32-amino-acid polypeptide hormone, is secreted from the parafollicular cells of the mammalian thyroid gland. CT receptor (CT-R) is a G protein–coupled cell surface receptor that expressed in osteoclasts, renal, and neural cells (10). The activity of CT is enhanced in calcium excretion from kidney whereas reduced in osteoclast-mediated bone reabsorption (11, 12).

Apart from the above mentioned, CT can reduce the serum calcium in hypercalcemia and increase bone accumulation in osteoporosis. In addition, CT-R in central nervous system (CNS) is highly concentrated not only in hypothalamus, but also in other regions including the PAG, raphe nuclei, and

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the areas which are thought to be crucial to nociception (13). Other physiological functions of CT including analgesia, inhibition of appetite and gastric acid secretion are also modulated by CT-R in the CNS (14, 15). The analgesic effect of CT has been proved in clinical study, especially in patients with vertebral fractures associated with osteoporosis (16) as well as cancer-related pain (17).

In this study, we proposed that CT could attenuate hyperalgesia in the midbrain PAG in chronic constriction injury rats. Furthermore, we also investigated the role of CT-R in the hyperalgesia in midbrain PAG. Here, we aimed to uncover the mechanism of hyperalgesia and to provide some experimental evidence for the treatment of bone pain.

Materials and Methods

Experimental animals and grouping

Forty-eight male Sprague–Dawley (SD) rats (5 to 6 weeks of age, 190 ± 10 g) were obtained from the Laboratory Animal Center of the Third Military Medical University (Chongqing, China). Salmon calcitonin (sCT) was purchased from Novartis Pharmaceutical Co., Ltd (Beijing, China). The animals were raised in a standard animal room at 25 ± 1˚C and received a 12 hr light /12 hr dark cycle. They had free access to food and water. All behavioural tests were performed between 09:00 and 14:00. This work was in accordance with the ethical guidelines of international association for study of pain (18), and the animal experiments were also approved by the ethical committee of Southwest Hospital.

The animals were randomly divided into six groups including control, sham, chronic constriction injury (CCI), CCI plus low-dose sCT, CCI plus middle-dose sCT, and CCI plus high-dose sCT groups (eight 8, 10, 12, and 14 after the CCI. All the surgeries were performed under anesthesia by 4% choral hydrate (10 ml/ kg, IP). Guide cannulas were placed in the PAG of the rats in CCI plus middle-dose sCT, and CCI plus high-dose sCT groups at the fifth day. The PAG microinjection was performed daily from 7 to 14 day. At the fourteenth day, the animals were sacrificed and the PAG was removed for the following analysis.

Induction of neuropathic pain model in rats

The neuropathic pain model was induced by chronic constriction injury. Briefly, the animals were anaesthetized after an intraperitoneal injection of 4% chloral hydratea (10 ml/kg). The right sciatic nerve was exposed at the mid-thigh level. Four 4/0 chromic gut sutures were tied loosely around and let a 1-mm interval for the nerve, and they were proximal to sciatic trifurcation (19). Then the wound was closed and treated with penicillin locally on the surface. The animals were conscious before they were transferred to the cages. The sciatic nerves of the animals in the sham group were also exposed except for the constriction.

Rat's head was fixated in a stereotaxic frame after an anaesthesia by 4% chloral hydrate (10 ml/ kg, IP). A 32-gauge stainless steel microinjection cannula (1.0-mm longer than the guide cannula) was attached to a 5-µl Hamilton syringe (Hamilton Company, Reno, NE, USA) and a PE-50 tube (Intramedic, Sparks, MD, USA), was inserted through the guide cannula while the rat was gently restrained. Either sCT (2.5, 5.0, 7.5 ng/ 0.5 µl) or normal saline was then injected through the microinjection cannula for more than 2 min. The microinjection cannula was left after an additional 30 sec to ensure a complete diffusion of the drug. Successful infusion was confirmed by monitoring the movement of small air bubbles during the injection procedure (Figure 1).

0.3 µl of Evans Blue (0.5%) was used for staining the injection site through the guider. The whole brains were removed and immersed in 10% formalin for the following histological analysis. 30-µm frozen sections were prepared, mounted, and stained for the identification of the microscopic injection site. Microinjection sites were verified by histological analysis and were plotted on coronal maps. Only those rats whose microinjection sites located within the PAG were used for the following analysis (Figure 1).

Behavioural test

Inclined plane test

The inclined plane test was performed to assess

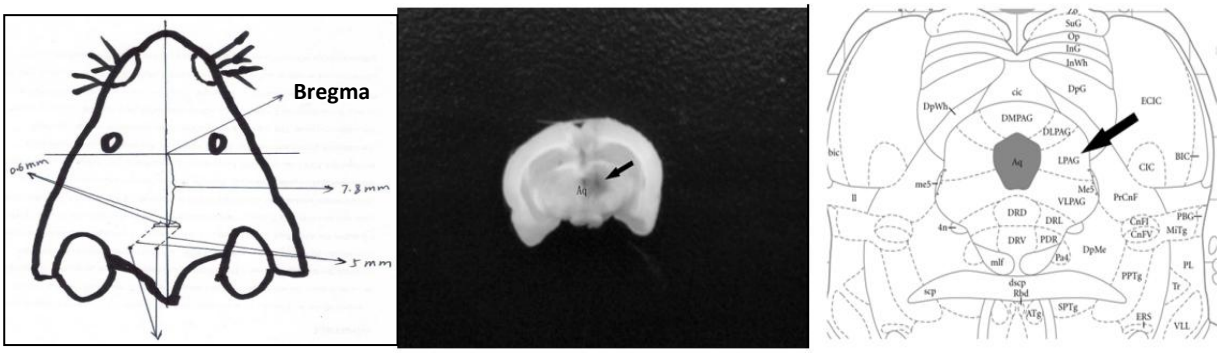

Figure 1. Microinjection site and identification of the injection site

baseline muscular strength and proprioception using a sliding apparatus (Medical Agent Co. Ltd., Kyoto, Japan) as described previously (20). Each rat was placed on a steel plate, which was inclined at 30°, and the angle of the plate was increased at a rate of 2˚ per sec. The maximum inclination was confirmed when a rat could maintain itself on the plane for at least 5 sec.

Hot plate test

To evaluate thermal nociception, the hot plate test was performed by placing the rat on a 52˚C plate (Biomedical Engineering Research Institute of Chinese Academy of Medical Sciences, Beijing, China) and measuring the thermal withdrawal latency (TWL) for licking a hind paw or jumping (21). The experiment was initiated after an adaptation for 30 min. The time interval between each experiment was 5 min. A 60-sec cutoff time was used to minimize tissue damage. The latency time was considered as the time between placing the mice on hot plate and the time when mice licked their fore and hind paws or jumped from the plate. Animals presenting baseline latencies higher than 20 sec were excluded (22).

Von Frey monofilaments test

Mechanical nociceptive threshold was measured using calibrated von Frey filaments (North Coast Medical, Morgan Hill, CA) as described previously (23). Animals were placed individually on a wire mesh floor of a plexiglass cage (20 cm × 20 cm × 25 cm). Cages were placed high enough so that the experimenter could access to the bottom of the cage freely. A set of von Frey monofilaments with bended forces ranging from 1.04 to 63.2 g were applied in ascending order perpendicular to the mid-plantar surface of the right hind paw.

The von Frey filament was held in the same position with enough force to cause a slight bend. Response to the touch of the exquisite withdrawal, such as licking the paw, transient vocalization, jumping, shaking the paw, biting at the probe or the stimulated paw response, was considered positive. In the absence of a paw withdrawal response, a thicker hair corresponding to a stronger stimulus was chosen. For each paw, a von Frey hair was applied five times and the interval was 5 sec, and response for more than three times in five individual experiments was considered positive.

Immunohistochemistry staining of CT-R in the PAG

The rats were anesthetized with 4% choral hydrate solution (10 ml/ kg, IP) and then 150 ml of normal saline was perfused with the ascending aorta followed by another perfusion of 200 ml of 0.2 M phosphate buffer (PB) containing 4% paraformaldehyde. Brain tissues were removed and post-fixed by an immersion in the same fixative overnight, and then post-fixed in 0.1 M phosphate-buffered saline (PBS) containing 30% sucrose at 4˚C for 48 to 72 hr. After stored at – 80˚C with embedding media (McMormick Co., USA) for 3 h, the 30-μm PAG sections were prepared using a frozen slicer.

After an incubation with 0.01 M PBS containing 3% H_2O_2 followed by 0.1 M PBS containing 3% equine serum albumin (BSA), the sections were co-incubated with rabbit polyclonal antibody against CT-R (1: 400) (UCL, USA). After incubation for 1 hr at 37˚C and overnight at 4˚C, the sections were incubated with a biotinylated secondary antibody (1: 400) (Beijing Zhongshan Biotech Co, Beijing, China) for 1 h at 37˚C. Subsequently, a horseradish enzyme HRP-avidin tertiary antibody (1: 200) (Beijing Zhongshan Biotech Co, Beijing, China) was added. Then the sections were stained by 0.05% diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, St. Louis, MO, USA), 0.25% nickel ammonium sulfate (DAB-Ni) (Sigma-Aldrich, St. Louis, MO, USA) in PBS containing 0.03% H_2O_2 for 5 min, then the sections were dehydrated through an ascending series of alcohol solutions and xylene. Finally, the sections were imaged using a laser scanning microscope (Olympus Fluoview FV1000, Japan). The absence of cross-reactivity was confirmed by a single-labeled control preparation.

Western blot analysis of CT-R protein in the PAG

The rats were decapitated by cervical dislocation at day 14 after the CCI, and the PAG tissue was removed rapidly. The tissue was homogenated in 400 μl pre-chilled lysis buffer containing 500 mM Tris-HCl (PH=7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 0.02% sodium azide, and then centrifuged at 3000 × g for 10 to 15 min. The supernatants were collected and further centrifuged at 20,000 × g for 30 min. Protein determination was performed by Bradford method (24).

10 to 20 μg of total protein was separated on 11% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS–PAGE) and then transferred onto nitrocellulose membrane at 70 V for 1 hr. Nonspecific binding sites were blocked with 6% nonfat milk for 2 hr at room temperature, and then the blots were incubated overnight at 4˚C with primary antibodies against CT-R (1:200) (UCL, USA) and β-actin (1:2000) (Calbiochem, Germany), respectively. Then a HRP-conjugated secondary antibody (1:5000) (Sigma, USA) was added and co-incubated for 1 h at room temperature. The blotted membrane was detected by enhanced chemiluminescence method with SuperSignal® West Pico Chemiluminescent Substrate (Thermo SCIENTIFIC, Rockford, IL, USA) and captured on X-ray film. The densitometry assay was performed using UVP's Gel Documentation SYSTEMgds8000 and Quantity One software (Bio-Rad, USA).

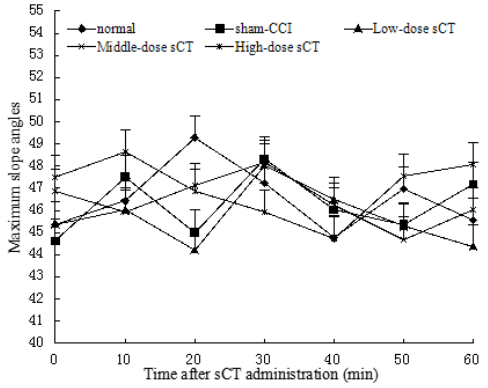

Figure 2. Effect of sCT microinjection in the PAG on maximum inclined angle. sCT (2.5, 5.0, and 7.5 ng) or an equal volume of NS was microinjected into the PAG at the initiation of the experiment. The inclined angle maintained by the animals before the injection served as a control. (Mean ± SD, n=8)

Histological verification for PAG

Evans blue 2% was injected into PAG through micro- injector after experiment. The injection site was determined by comparing with rat brain atlas (25). Only the rat with injection site located at LPAG was included in the statistical analysis (Figure 1).

Statistical analysis

All the data was expressed as mean±SD. The differences among groups were analyzed using SPSS 11.0 software (Chicago, USA). Statistical differences between two groups were analyzed by *t* test and differences between multiple groups of data were assessed by univariate ANOVA. *P*-values of less than 0.05 were considered statistically significant.

Results

Alternations of motor function after CCI

The result of inclined plate revealed that there was no significant difference in the maximum maintained angle in different groups ($P > 0.05$) (Figure 2).

Alternations of TWL and MWT after CCI

The TWL result demonstrated that the TWL before the surgery (19.6 ± 1.19 sec) was significantly longer than that at day 2 (12.5 ± 1.60 sec), and day 14 (7.75 ± 0.89 sec), respectively ($P < 0.01$) (Figure 3).

Similar to the result of TWL, the MWT before the surgery (20.5 ± 1.17 g) was more than that at day 2 (10.7 ± 1.25 g) and day 14 (4.1 ± 0.88 g) ($P < 0.01$; $P < 0.01$), respectively. However, the TWL and MWT in the normal and sham groups remained unchanged throughout the whole experiment (Figure 3).

Analgesic effect of sCT on CCI

Salmon calcitonin was microinjected into the PAG once daily from day 7 to 14. In the present study, the TWL and MWT in the control CCI group were not significantly altered (*$P > 0.05$). After microinjection with sCT at different doses (2.5 ng/0.5 µl, 5.0 ng/1.0 µl, and 7.5 ng/1.5 µl), the TWL at day 14 was

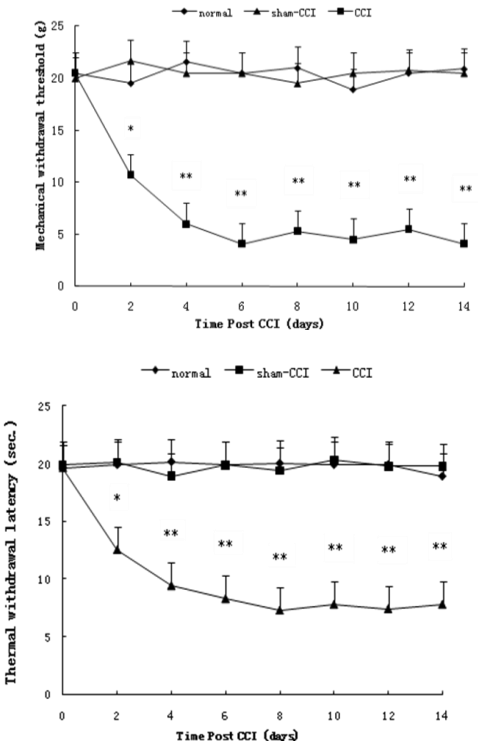

Figure 3. CCI induced thermal and mechanical hyperalgesia in rats ($\bar{X}$ **±s,** n=8)**.** From day 2 after CCI, the values of TWL and MWT in the CCI group were significantly lower than those in the normal group. *$P < 0.05$, **$P < 0.01$ versus the normal group

significantly increased compared to that at day 7 (12.3 ± 1.19 sec versus 6.1 ± 1.03 sec, 14.3 ± 1.38 sec versus 5.9 ± 0.87 sec, and 16.1 ± 1.26 sec versus 6.3 ± 0.92 sec, respectively) (*$P < 0.05$). In addition, the MWT at day 14 was markedly elevated compared to that at day 7 (7.7 ± 1.07 g versus 4.1 ± 0.91 g, 9.0 ± 1.41 g versus 4.1 ± 0.91 g, and 10.7 ± 1.33 g versus 4.1 ± 0.91 g) (*$P < 0.05$) (Figure 4).

Distribution of CT-R in the PAG

The staining of CT-R in PAG of the CCI rats was stronger and the distribution was larger than that of in the normal and sham group, respectively. The CT-R expressed in the neurons of the PAG. No staining was observed in the negative control group. After the microinjection of sCT for 7 day, the staining of CT-R was significantly stronger than that of in the CCI group (Figure 5).

CCI and sCT microinjection up-regulated CT-R protein expression in the PAG

The expression level of CT-R protein in the CCI group was significantly higher than that in the normal group and sham group, respectively ($P < 0.01$; $P < 0.01$). Meanwhile, sCT efficiently up-regulated the CT-R protein expression in a dose-dependent manner (Figure 6).

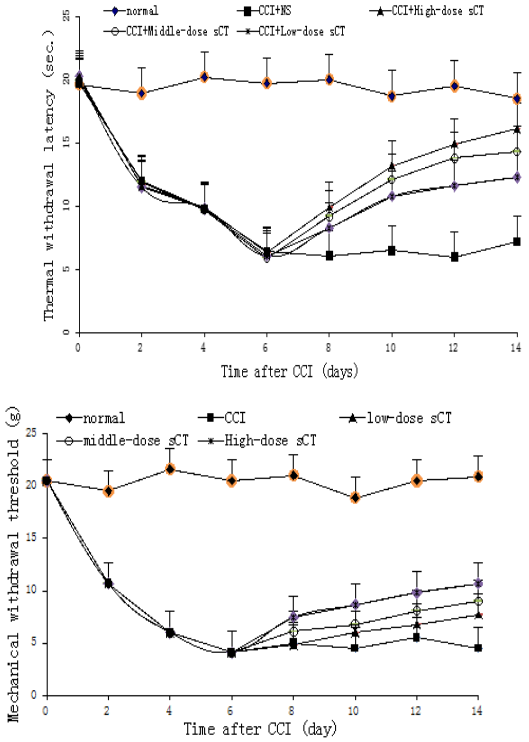

Figure 4. Effects of sCT microinjection on TWL and MWT in the PAG of CCI rats (mean ± SD, n=8). sCT (2.5, 5.0, and 7.5 ng) or an equal volume of NS was microinjected into the PAG once daily from day 7 to day 14. *P< 0.05 versus the same group at day 7

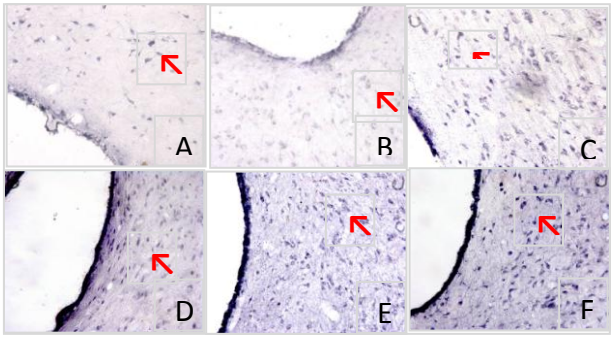

Figure 5. Immunochemistry staining of CT-R in the PAG. A normal group; B sham-CCI group; C CCI group; D CCI+Low-dose sCT group; E CCI+Middle-dose sCT group; F CCI+High-dose sCT group. Arrows indicate the positive CT-R cells. (Original magnification ×40)

Discussion

Pain is a multidimensional phenomenon that encompasses sensory-discriminative, affective-motivational, and cognitive-emotional components mediated by different mechanisms and processed in a neural network (26). Peripheral nerve injury sometimes results in a chronic neuropathic pain syndrome characterized by hyperalgesia, spontaneous pain, radioactive pain, and nociceptive pain in response to normally innocuous stimulation. Most of these symptoms have been recently documented in a rodent model of peripheral mononeuropathy induced by a loose ligation of common sciatic nerve in rats, which was also described as a chronic constrictive injury (CCI) (19). Vertebral fragility fractures are usually accompanied by compression of nerve root or spinal cord, resulting in peripheral nerve injury which commonly occurs in osteoporosis patients and is similar to animal model of CCI (27). Because it is difficult to create animal model of osteoporotic pain, we employed CCI rat model to investigate the central analgesia mechanism of sCT, a common agent clinically utilized to manage many kind of pain such as neuropathic pain, migraine, mild lumbar stenosis , and pain caused by bone metastasis.

A considerable amount of clinical evidence displayed a good analgesic effect of CT (27). It has been speculated that this hormone influences generation of pain induction or pain-associated substances (28). However, the relationship between CT-induced analgesia and endocrine substances is still disputable. CT injection in the PAG has been reported to produce anti-nociception whereas systemic injection could not obtain the analgesic effect, suggesting CT exerts not peripheral but central effects (29, 30). Repeated systemic administration of CT also could achieve the antinociceptive effect (31). However, this effect is delayed since it is difficult for CT to penetrate blood-brain barrier. Naloxone failed to reverse sCT-induced analgesia, suggesting an opiate independent mechanism for this peptide in analgesia elicitation (32). We hypothesized that CT could increase the release of an excitatory neurotransmitter by promoting calcium influx in the CNS. Actually, the activation of CT-R, which mediated calcium influx and stimulation of membrane potential, might be an essential candidate for the effects mentioned above (33).

In this study, with neuropathic pain model of SD rats, the CT receptor protein level and expression location in the midbrain periaqueductal gray (PAG), a crucial site in endo-genous pain modulatory system, were evaluated by immunohistochemistry and Western blotting. We found a comparatively weaker CT-R specific immunoreactivity in the PAG in rats in the normal and sham groups, which was consistent with previous reports that the binding site of CT was present in the ventral and ventrolateral segments of the PAG (29, 32). The study also showed that rats in the CCI group had comparatively lower TWL and MWT than those in the normal and sham groups along with stronger and more extensive CT-R expression. Meanwhile, the CT-R immunoreactive cells were expressed in the neurons in the PAG, suggesting increased CT-R specific immunoreactivity after nerve constriction injury in the PAG. To our knowledge, PAG is a key region in the regulatory system for endogenous pain, and neurons activation in the PAG is capable of suppressing harmful inputs when dorsal horn neurons fail to respond and withdraw (34-36).

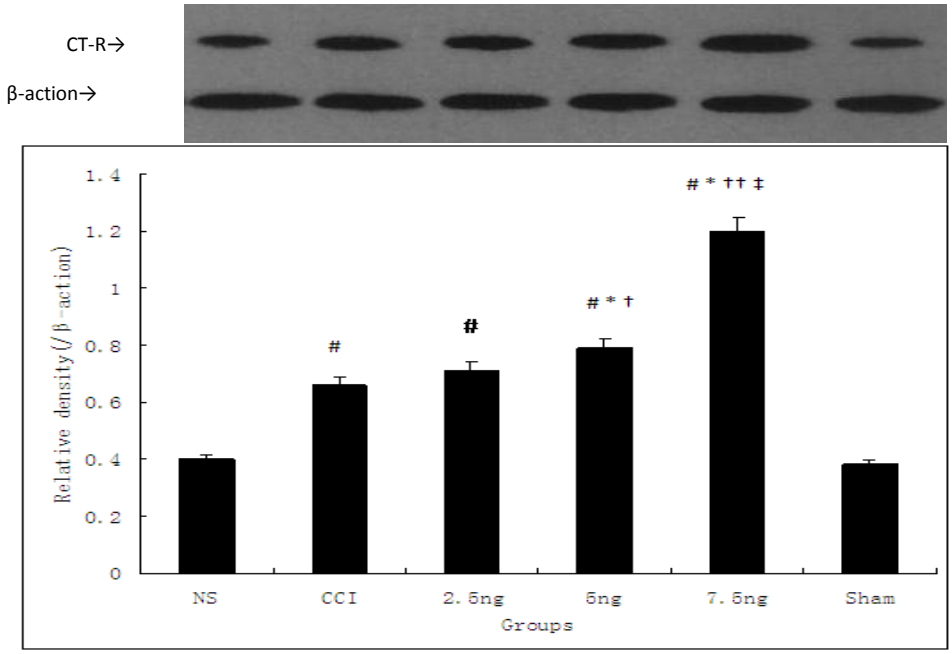

Figure 6. Effect of sCT on the expression of CT-R protein in PAG neurons of CCI rats. **T**he expression of CT-R in the CCI group was remarkably stronger than that of in the normal control group. Compared with the CCI group, the expression of CT-R protein in the middle and high sCT groups was significantly increased in a dose-dependent manner. Lane 1: normal group; Lane 2: CCI group; Lane 3: CCI+low-dose sCT group; Lane 4: CCI + middle-dose sCT group; Lane 5: CCI+ high-dose sCT group; Lane 6: Sham group; #*P*< 0.01 versus NS; **P*< 0.01 versus CCI; †*P*< 0.05, ††*P*< 0.01 versus sCI (2.5 ng); ‡ *P*< 0.05versus sCI (5.0 ng)

Salmon calcitoninis a kind of agent plays an important role against CT-R. In the following study, the effects of various doses of CT were examined. The results showed a higher pain threshold and a more extensive expression of CT-R protein after microinjection of sCT in the PAG. Moreover, the effect of sCT on CT-R upregualtion was in a dose-dependent manner, which was similar to the study of Aboufatima *et al* (37). These results suggest that CT receptors in the PAG play an inhibitory role in pain modulation, and CT exerts a marked therapeutic effect in relieving neuropathic pain in CCI rats, which may be related to its regulative effect on the expression of CT receptors in the PAG. In conclusion, CT receptors in the PAG are involved in the central nervous system antiociception effect of CT treatment for pain.

There are several methodological limitations in this study. Firstly, sCT is an agent not used generally for neuropathic pain but mainly for osteoporotic pain. Meanwhile, ovariectomy-induced bone pain was not discussed in this study. However, the pain induced by CCI is quite different from the clinical feature and osteoporotic pain. Secondly, in the present study, we used intracerebral injection, despite the use of nasal spray or subcutaneous injection clinically. It may amplify the analgesic effect of CT and the response of brain. Thirdly, the central mechanism of CT was not fully clarified in the present study. In addition, further study is needed to be investigated whether CT-R is activated in PAG neurons as well as the relationship between CT-R and pain-related substances such as opioid, ATP, β-endorphin, and glutamate (38-41).

Conclusion

In this study, we found that the increased expression of CT-R might be associated with hyperalgesia in CCI rats, and sCT had an anti-nociceptive effect on CCI rat through upregulation of CT-R in the PAG.

Acknowledgment

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Conflict of interests

No potential conflicts of interest relevant to this article were declared.

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