

# Preliminary study of calcium homeostasis modulator 1 involved in trigeminal neuralgia

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## Research article

**Keywords:** trigeminal neuralgia; spinal trigeminal nucleus; calcium homeostasis modulator 1; ruthenium red

**Posted Date:** March 9th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-16441/v1>

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# Abstract

**Background:** The aim of this study was to observe the changes in the expression of calcium homeostasis modulator 1 (CALHM1) in the trigeminal nucleus of the trigeminal neuralgia (TN) rats with the infraorbital nerve-chronic constrictive nerve injury (ION-CCI) and to explore the role of CALHM1 in TN.

**Methods:** Thirty SD rats were randomly assigned to 5 groups, namely normal control group (Control group), sham operation group (Sham group), TN model group (ION-CCI group), ruthenium red treatment group (RuR group) and control group of ruthenium red (NS group), with 6 rats in each group. An animal model was established by loosely ligating the rat's infraorbital nerve with a chrome gut in the ION-CCI group, while mice in the sham group were only exposed to the nerve and did not receive ligature. The rats in the RuR group were intraperitoneally injected with 0.5 mg/kg of CALHM1 inhibitor ruthenium red on the 9th day after the infraorbital nerve ligation, while the rats in NS group were intraperitoneally injected with an equal volume of normal saline on the 9th day after surgery. All experimental rats were tested for pain behavior 1 day before surgery, 1, 3, 5, 7, 9, 11 and 14 days after surgery, including the mechanical pain threshold of VonFrey filament in the trigeminal innervated skin area and number of faces captured in video recording. The expression of CALHM1 in the trigeminal spinal nucleus was detected on the 15th day after operation in all experimental groups.

**Results:** The expression of CALHM1 in the trigeminal spinal nucleus of the ION-CCI group was significantly higher than that of control group and sham group on the 3rd and 15th day after modeling. The intraperitoneal injection of CALHM1 inhibitor ruthenium red increased the mechanical pain threshold of ION-CCI rats and significantly reduced the number of scratches, but did not change the expression of CALHM1 in the trigeminal spinal nucleus.

**Conclusion:** The expression of CALHM1 protein in the trigeminal spinal nucleus is involved in the central sensitization of TN pain, which can be induced by elevated expression. Moreover, the hyperalgesia can be improved by using CALHM1 inhibitor.

## Background

Trigeminal nerve injury or infection caused by maxillofacial intractable pain is a common clinical problem. TN is a form of neuropathic pain with a major pathological change in the loss of trigeminal root axons and demyelination. Clinically, it often characterized by sudden electric shock-like burst pain and tactile hyperalgesia, and because of repeated pain, the pain is severe and difficult to cure, which seriously affects the quality of life of patients(1). Based on the above theory, a chronic constriction injury of the infraorbital nerve (ION-CCI) model was established in the 1990s, which is the most commonly used model in the study of trigeminal neuralgia(2). The model is easy to operate and has a high modeling rate, which can better simulate the clinical manifestations of human trigeminal neuralgia(3, 4).

CALHMI is a cell surface calcium channel expressed in brain neurons. Studies(5) have shown that CALHM1 controls intracellular calcium signaling, which reduces the resistance of ion influx in sensory

neurons and enhances the ion influx of neurons. Thus, the excitability of neuronal cells is dramatically enhanced(6). Whether CALHM1 is involved in the occurrence of TN has not been reported in the literature. The aim of this study was to investigate the role of CALHM1 in TN in the trigeminal spinal nucleus of rats with ION-CCI.

## Methods

### Experimental animal

All animals were purchased from the Experimental Animal Center of Central South University. The experimental procedures were approved by the Animal Management and Use Committee of the Third Xiangya Hospital of Central South University, and was in compliance with the Guide for the Care and Use of Laboratory Animals published by the Chinese National Institute of Health. Before any experiment, adult male SD rats (200-220 g, a total of 30) were raised and maintained under standard laboratory conditions, and allowed to acclimate in the cage for 12/12 hours under light-dark cycle, and they were able to eat and drink at any time.

### Construction of a trigeminal pathological pain model

The rats tested were subjected to relevant adaptive training 2 weeks before the start of the experiment, and rats with stable mood expression against VonFrey hair were selected for modeling. The animals were fasted for 12 h before the experiment, but had access to water ad libitum. They were anesthetized with an intraperitoneal injection of Pentobarbital (3%, 30mg/kg). The unilateral infraorbital nerve was loosely ligated as described by Kernisant(7) to establish a trigeminal pathological pain model. The infraorbital nerve was loosely ligated with absorbable chromium gut. The ligation standard was that the diameter of the nerve was slightly thinned after ligation under the operating microscope, but the conduction could not be completely blocked, and the blood circulation of the epicardium remained unobstructed.

### Experimental grouping

Thirty SD rats were randomly divided into control group, sham group, ION-CCI group, NS group (ION-CCI + normal saline) and RuR group (ION-CCI + ruthenium red), with 6 rats in each group. ION-CCI was induced by ligation of the unilateral infraorbital nerve with chromium gut loosening to establish an animal model of TN. The rats in the control group were not treated, and the surgical procedure of rats in sham group were the same as the ION-CCI group except that the infraorbital nerve was not ligated, that is, only the nerves were exposed and not ligated. On the 9th day after surgery, rats in the RuR group were intraperitoneally injected with CALHM1 inhibitor ruthenium red 0.5mg/(kg•rat•day), while the rats in the NS group was injected with an equal volume of physiological saline. In control group, sham group and ION-CCI group, mice were euthanized by three times the anesthetic dose of pentobarbital after behavioral tests. In NS group and RuR group, mice were anesthetized by Pentobarbital (3%, 30mg/kg) and euthanized by obtaining brain tissue.

## **Pain behavior test**

Determination of mechanical pain threshold: All experimental rats should be acclimated to the environment for two weeks in advance, and pain behavioral measurements were performed 1 day before surgery (ION surgery or sham surgery) and 1, 3, 5, 7, 9, 11, 14 days after treatment. According to Vos BP et al.(8), the Von Frey filament is placed near the center of the whisker pad so that the filament is slightly curved. Stimulation of each intensity was stimulated at least 3 times from the minimum stimulation intensity, and the shortest interval between each 2 stimulations was 30 seconds, which is considered a positive reaction if one or more of the following behaviors occur: (1) Rapid withdrawal response in rats; (2) Escape or attack response; (3) The asymmetrical facial trimming behavior is characterized by more than 3 consecutive strokes of the stimulated facial area. The minimum value of the filament strength that causes a positive reaction in the rat is the mechanical pain threshold of the surgical side whisker pad. Measurements were not performed in the order of grouping, and the order of placement of rats was disrupted, and random measurements were made by those who did not understand the experimental group of rats to reduce experimental errors.

Video recording of the number of scratches: According to Spradley(9), rats were placed in a colorless transparent glass lattice that did not interfere with rats' random movement. Rats were used to adapt to the environment of the lattice in advance, and they were obtained to move freely for about an hour before they began video recording. The video observation lasted about 30 minutes. The observers were not informed of the grouping before the experiment, and the number of scratches of each group of rats at the corresponding time points (1 day before surgery and 1, 3, 5, 7, 9, 11, and 14 days after surgery) was recorded. Keep quiet during the recording process so as not to scare the rats into affecting the experimental results.

## **Western blotting analysis**

On the 15th day after operation, the trigeminal nucleus of the rat trigeminal spinal nucleus was removed under low temperature and aseptically under the direction of the brain stereotaxic instrument. The lysate containing the protease inhibitor was added, and the tissue was homogenized and placed in 4 °C. After centrifugation at 12,000 rpm for 15 minutes in a centrifuge, the supernatant was taken and the protein content of the sample was determined by (Bicinchoninic acid) BCA quantification. The protein was then denatured by adding a loading buffer and drying at 100 ° C for 5 min. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransformation, the protein was transferred to a polyvinylidene fluoride (PVDF) membrane and blocked for 2 hours with 5% skim milk. Rabbit anti-CALHM1 primary antibody (1:1 000) diluted with 5% TBST was added and left overnight at 4 °C. After washing the membrane, the mouse secondary antibody (1:5000) was separately added, and the membrane was then incubated at 37°C for 1 h, and then subjected to ECL coloration and exposure. Semi-quantitative analysis was performed using  $\beta$ -actin as an internal control.

## **Statistical processing**

All data were expressed as mean  $\pm$  standard deviation, and the results of western blot were analyzed using Image J for gray scale analysis. Two-Way ANOVA was used to compare the mechanical pain threshold and video behavioral results of each group of rats, and comparison between groups was performed at the same time point. The difference of gray value of each group was analyzed by one-way ANOVA and then compared by Tukey method. The difference was statistically significant ( $P < 0.05$ ). The data were analyzed and plotted by Graphpad Prism 6 software.

## Results

### Test results of pain behavior

#### **The looseness of the unilateral infraorbital nerve of the ligated rat with the chrome gut can cause hyperalgesia in the rat's mouth and face.**

Mechanical pain sensitivity tests were performed on the control group, sham group and ION-CCI group at preoperative and postoperative specific time points. Consequently, the pain behavioral results in this study showed that rats with ligation of the infraorbital nerve showed painful dullness from the first day to the third day after surgery, showing a brief and sharp increase in the threshold of mechanical pain, and the level was significantly higher than control group and sham group (Fig. 1A,  $P < 0.01$ ). By the fifth day, it slowly dropped to the same level as the control group and the sham group. Meanwhile, rats with trigeminal neuralgia continued to show pain sensitivity until the 9th day in this study ( $P < 0.05$ ), and the mechanical pain threshold reached the lowest at 14 days after surgery ( $P < 0.01$ ). The mechanical pain threshold on the operation side of the control group and the sham group was not significantly different before and after surgery, and there was no statistical difference. The video behavioral results of the control group, the sham group and the ION-CCI group showed that the number of scratches on the first and third days after surgery in the ION-CCI group and the sham group was significantly higher than that in the control group (Fig. 1B,  $P < 0.01$ ,  $P < 0.01$ ). The number of scratches in the sham group gradually decreased from the third day to the same level as that in the control group, and the number of scratches in the ION-CCI group decreased slowly from the third day until the 7th day. And then the number of scratches in the ION-CCI group was significantly higher than that of the control group and the sham group ( $P < 0.01$ ), and it increased slowly and continued until the 14th day. The susceptibility of the surgical side of the ION-CCI group ligated to the supraorbital nerve was significantly higher than that of the other two groups, thereby judging that the rat model was successfully established in ION-CCI group.

#### **Injection of CALHM1 channel inhibitor ruthenium red can improve hyperalgesia in TN rats**

To confirm whether elevation of CALHM1 in the trigeminal spinal nucleus caused by ION-CCI mediates mouth and facial hyperalgesia in rats with trigeminal neuralgia, the CALHM1 channel inhibitor ruthenium red (0.5mg/kg/rat/day) was intraperitoneally injected for 6 consecutive days when the hyperalgesia of the rats in which the infraorbital nerve was ligated occurred, that is, on the 9th day, and the NS group was injected with an equal volume of physiological saline as a control. At the same time, mechanical pain threshold and video behavioral testing were performed every other day. Western blot analysis was

selected on the 15th day after the last injection to verify whether the treatment of ruthenium red could block or alleviate the high-sensitivity state of oral and facial behavior and the expression level of CALHM1 caused by the injury. As shown in Fig. 2, the tendency of threshold of the intraoperative lateral tentacle pad and the surrounding mechanical pain threshold as well as the number of scratches in the RuR group were consistent with the ION-CCI group and the NS group in the first 9 days after surgery, and there was no statistical difference. After the injection of the drug, the mechanical pain threshold of the RuR group no longer decreased significantly on the 11th to 14th day after surgery, but remained at a certain level, which was significantly different from the ION-CCI group and the NS group. The number of scratches in the RuR group gradually decreased on the 9th day after the operation, that is, on the first day after administration, and decreased to the lowest level on the 14th day. By contrast, the number of scratches in the ION-CCI group and the NS group gradually increased to the 14th day, showing a trend opposite to that of the RuR group, which was manifested as hyperpathia. That is to say, on the 11th and 14th day after intraperitoneal injection of ruthenium red in ION-CCI rats, the mechanical pain threshold and the number of scratches on the surgical side of the RuR group were significantly different from those of the ION-CCI group and the NS group. In summary, the pain behavior of the RuR group was significantly reduced compared with the other two groups, which improved the hyperalgesia status of rats with TN.

### **The expression of CALHM1 in the trigeminal spinal nucleus of the rats after ION-CCI was increased.**

We hypothesized that if the dysregulation of CALHM1 caused by infraorbital nerve injury after ION-CCI is a key factor in TN, thus the CALHM1 imbalance caused by injury should be related to the occurrence of the state of oral and facial pain. To testify this hypothesis, we examined the expression of CALHM1 in the lateral trigeminal nucleus of the rats in each group including TN with ION-CCI and also confirmed whether there was any change in CALHM1 expression between ION-CCI and other control groups. As shown in Figure 3, CALHM1 was expressed in the normal trigeminal nucleus. In comparison with the sham group, CALHM1 began to increase significantly on day 3 in ION-CCI rats undergoing infraorbital nerve ligation, and reached the peak on the 15th day, which was consistent with the hyperpathia of ION-CCI rats and NS rats on day 15 than that of control and sham groups. The consistency of the mechanical hyperalgesia state of the whisker pad and its surroundings as well as the increase in the expression of CALHM1 in the spinal nucleus caused by the infraorbital nerve ligation suggests that elevated CALHM1 in the trigeminal nucleus may play an important role in TN.

### **Injection of CALHM1 channel inhibitor ruthenium red failed to affect the expression level of CALHM1 in the spinal trigeminal nucleus of rats after ION-CCI**

As mentioned earlier, on the 9th day after ION-CCI, we injected CALHM1 inhibitor ruthenium red to rats that had just begun to develop facial hyperresponsiveness for 6 consecutive days, and on the 15th day, the injected rats were analyzed for CALHM1 expression levels in the trigeminal spinal nucleus. As demonstrated in Fig. 4, we found that the RuR group had a significant improvement in pain behavior compared to the NS control group and the ION-CCI group. At the same time, there was no statistical

difference in protein expression, and p values were 0.11 and 0.16, respectively, suggesting that ruthenium red improves hyperalgesia in TN rats by a pathway that does not affect CALHM1 expression.

## Discussions

There are many different opinions on the pathogenesis of TN. At present, the following views are basically recognized by scholars at home and abroad who have studied TN(10): Peripheral causes are important pathological basis of TN, and central factors are also indispensable. At present, the most widely used model in the study of TN is ION-CCI. The infraorbital nerve is one of the branches of the trigeminal nerve that is responsible for transmitting the sensation around the rat's tentacles. Therefore, in the trigeminal animal model, it is recognized that the more reasonable part of the research mechanism is around the tentacle pad, and it is theoretically reliable to mold at the corresponding position. Based on this principle, we constructed an experimental study of the state of simulated TN.

The pain behavioral results in this study showed that rats with ligation of the infraorbital nerve showed painful dullness from the first day to the third day after surgery, showing a brief and sharp increase in the threshold of mechanical pain, and the level was significantly higher than control group and sham group. By the fifth day, it slowly dropped to the same level as the control group and the sham group. This result is consistent with the findings of Kajander KC and other studies(11), which are considered to be caused by the trigeminal nerve conduction impulse of the rat after ligation. After recording the activity of primary afferent axons in rats with sciatic nerve ligation, it was found by Kajander KC et al. that the proportion of axons that could not reach the nerve impulse axon ratio of the injured site on the first postoperative day was: 85% A $\beta$  fiber and 55% A $\delta$  fiber. By day 3, the proportion of affected A $\beta$  fibers and A $\delta$  fibers increased to 89% and 87%, which is consistent with the behavioral increase in the mechanical pain threshold of rats on days 1–3. In this study, TN rats continued to show pain sensitivity on the 9th day, and the mechanical pain threshold reached the lowest level at 14 days after surgery. This result is basically consistent with the findings reported by Vos BP et al (8).

It was found in the study that the expression of CALHM1 in the trigeminal nucleus of the ION-CCI group was significantly increased on day 14 compared with the control group or sham group. Compared with the ION-CCI/NS group, RuR group showed a significant improvement in hyperalgesia status in TN rats, suggesting that CALHM1 may be involved in the development of pain behavior in TN rats. In recent years, AD-related studies have shown that CALHM1 can affect neuronal signaling by affecting the expression of MEK, ERK, RSK and MSK, or lead to enhanced excitability of neurons, which plays an important role in synchronized discharge<sup>[3, 9]</sup>. WB analysis of neurons in the spinal dorsal horn of rats with painful diabetic neuropathy (PDN) analyzed by Wenjie Liu(12) showed that the expression level of CALHM1 was significantly higher than that of the control group, and the corresponding mRNA expression level was negatively correlated with the 50% reduction threshold. It is believed that CALHM1 is associated with the occurrence of PDN, which is consistent with our experimental results, suggesting that CALHM1 plays an important role in the pathogenesis of neuropathic pain. However, WB analysis of rats in RuR group showed that the expression level of CALHM1 was not statistically different from that of NS group and

ION-CCI group, while there was a statistically significant difference between the RuR group and the ION-CCI/NS group in terms of pain-related behavioral indicators in rats. And once the TN rats were intraperitoneally injected with ruthenium red, their hyperalgesia would be improved, and the mechanical pain threshold of the rats would also be elevated, with the corresponding decrease of the number of scratches. The cause of pain behavioral changes cannot be explained from the aspect of CALHM1 expression. Currently, no specific compound can regulate the expression level of CALHM1(13, 14). And CALHM1 acts as an ion channel to mediate ion influx, while non-specific inorganic dyes such as ruthenium red and  $Zn^{2+}$  can eliminate the action of CALHM1 by completely blocking the permeability of CALHM1. In the study of AD, Dreses et al.(15) found that CALHM1 which acts as a channel that penetrates  $Ca^{2+}$  can activate the MEK1/2, EPK1/2, RSK1/2/3 and MSK1 signaling pathways in neurons to regulate neuronal excitability. The hippocampal HT-22 cell line transduced with CALHM1 was incubated in a nutrient solution supplemented with ruthenium red and found that CALHM1-mediated  $Ca^{2+}$  influx was completely blocked. Moreover, its activation of the neuronal MEK/ERK/RSK/MSK signaling pathway has completely disappeared(15). This result suggests that the role of CALHM1 in neuronal signaling is completely blocked by ruthenium red. This result is not accidental. The accumulation of  $A\beta$  in the brain is considered to be a characteristic lesion of Alzheimer's disease, and CALHM1 can inhibit the accumulation of  $A\beta$  by affecting the clearance of  $A\beta$ (13). After incubation with ruthenium red in cells transduced with CALHM1, it was found that permeability of CALHM1 was completely inhibited, accompanied by the elimination of CALHM1 inhibition of  $A\beta$ , indicating that the application of ruthenium blocked the effect of CALHM1 on  $A\beta$ (13). In addition, in the model of cerebral hypoxia injury caused by clamping the middle cerebral artery, knocking out the CALHM1 gene or by intraperitoneal injection of ruthenium red can significantly reduce the infarct size(16). These results suggested that normal function of CALHM1 channel is a prerequisite for CALHM1 function. As an inhibitor of ion channel, ruthenium red can block the action of CALHM1 by inhibiting CALHM1 channel-mediated ion influx without affecting the expression level of CALHM1, which was consistent with our findings. Ruthenium red blocks the action of CALHM1 and improves the behavior of hyperalgesia in rats.

## Conclusion

The expression of CALHM1 protein in the trigeminal spinal nucleus is involved in the central sensitization of TN pain, which can be induced by elevated expression. Moreover, the hyperalgesia can be improved by using CALHM1 inhibitor. We believe that CALHM1 may play an important role in the changes of pain behavior in TN rats, which is expected to be a potential target for the treatment of TN.

## Abbreviations

calcium homeostasis modulator 1 (CALHM1)

trigeminal neuralgia (TN)

infraorbital nerve-chronic constrictive nerve injury (ION-CCI)

ruthenium red treatment group (RuR group)

normal saline (NS)

Bicinchoninic acid (BCA)

Tris-Buffered Saline Tween-20 (TBST)

Enhanced chemiluminescence (ECL)

painful diabetic neuropathy (PDN)

Sprague-Dawley (SD)

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

polyvinylidene fluoride (PVDF)

## **Declarations**

### **Ethics approval and consent to participate**

The experimental procedures were approved by the Animal Management and Use Committee of the Third Xiangya Hospital of Central South University.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Acknowledgement**

We would like to thank Animal experimental center of Xiangya Third Hospital and medical experimental center of Xiangya Third Hospital for technical assistance.

### **Funding**

This work was supported by the Hunan Provincial Natural Science Foundation of China (Grant No. 2019JJ40462). And the host of this project is involved in designing and supervising this study, analyzing and explaining data, as well as writing the manuscript.

### **Competing interests**

The authors declare that they have no competing interests.

### **Authors' contributions**

YL and KL: performed the experiments, collected and analyzed data, and wrote the manuscript. DH and XY: designed and supervised this study, analyzed and explained data, as well as wrote the manuscript. All authors read and approved the final manuscript.

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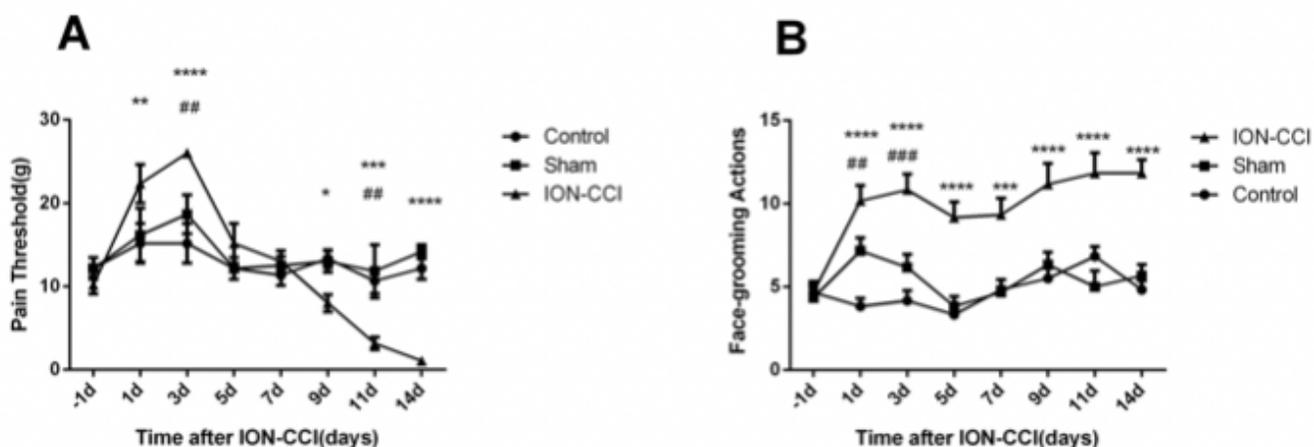
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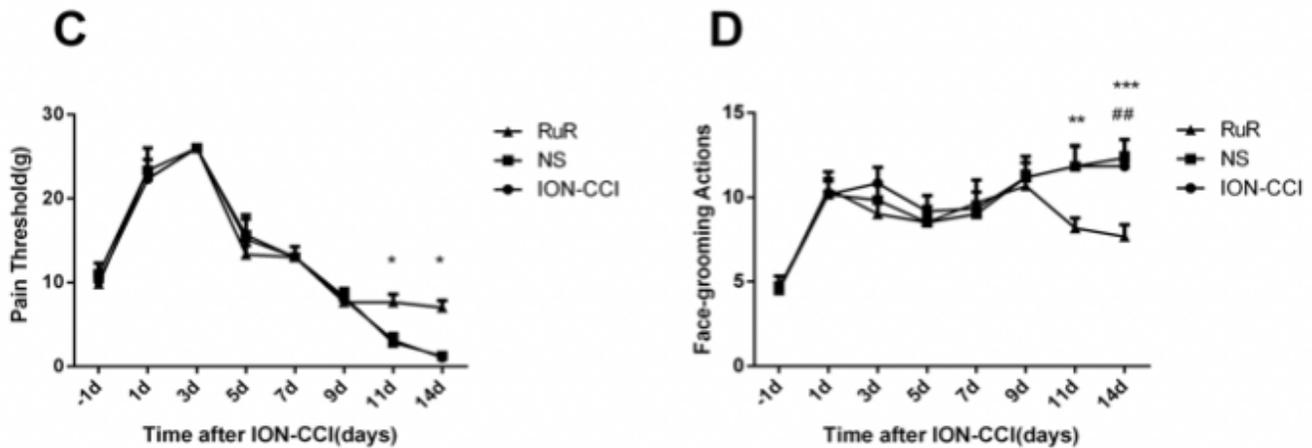
## Figures



**Figure 1**

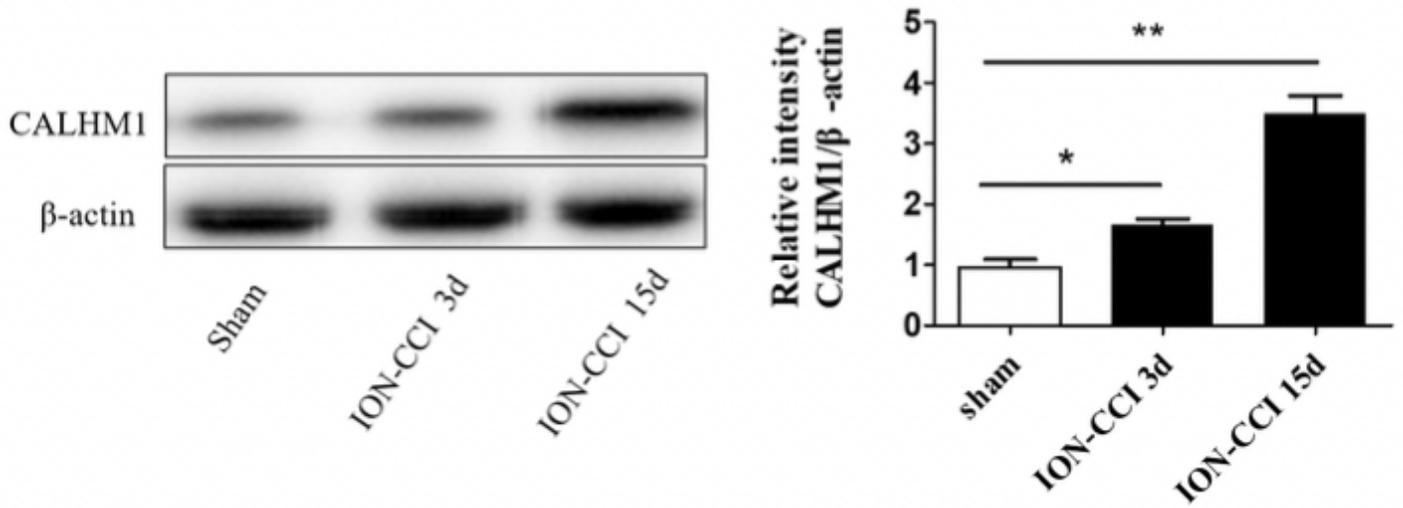
Results of Pain behavior test. Chronic constriction injury of the infraorbital nerve can lead to hyperalgesia in the lateral side of the rat. A. The threshold of the surgical side and the surrounding mechanical pain on

the 9th, 11th and 14th day after operation in the ION-CCI group were significantly lower than that of the control group and the sham group ( $n=6$ ,  $*P<0.05$ ,  $**P<0.01$  and  $##P<0.01$ ,  $***P<0.001$ ,  $****P<0.0001$ ). B. The number of face scratches on the 5th, 7th, 9th, 11th and 14th day after operation in the ION-CCI group was significantly higher than that in the control group and the sham group ( $***P<0.001$ ,  $****P<0.0001$ ).



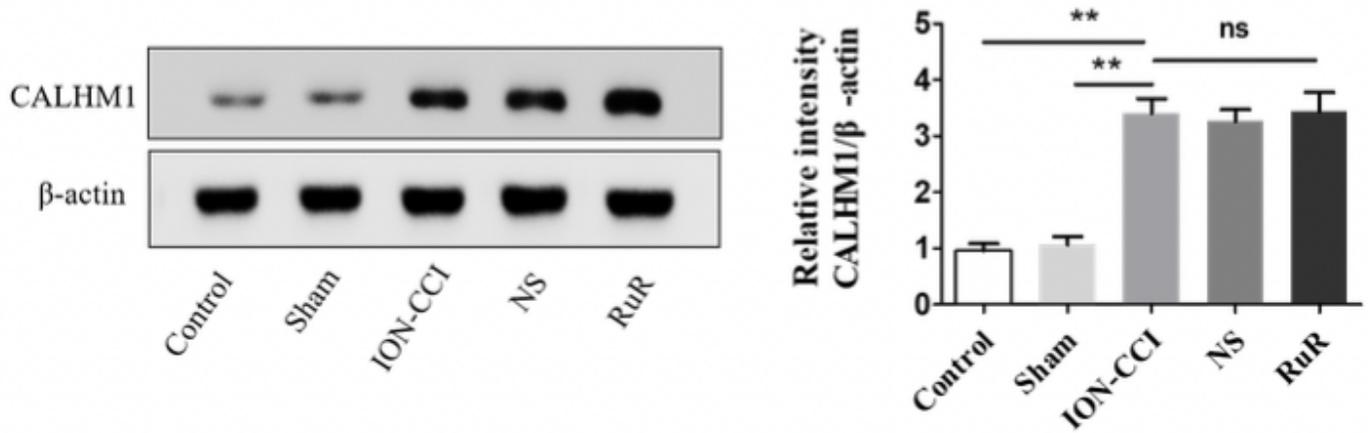
**Figure 3**

Results of Pain behavior test. The application of CALHM1 inhibitor ruthenium red can improve the mechanical pain threshold and reduce the number of scratches in TN rats. C. The threshold of the intraoperative lateral tentacle pad and the surrounding mechanical pain on the 11th and 14th day after surgery in the RuR group were significantly higher than those in the ION-CCI group and the NS group ( $n=6$ ,  $*P<0.05$ ), but there was no significant difference in the mechanical pain threshold between the ION-CCI group and the NS group. D. The number of face scratches on the 11th and 14th day after surgery in the RuR group was significantly lower than that in the ION-CCI group and the NS group ( $n=6$ ,  $**P<0.01$ ,  $##P<0.01$  and  $***P<0.001$ ). There was no significant difference in the number of face grabs between the ION-CCI group and the NS group.



**Figure 6**

The changes in protein (CALHM1) expression were confirmed by Western blot. Compared to the sham group, CALHM1 expression was significantly increased on the third day after ION-CCI surgery and further increased at 15th day (\* $P < 0.05$ , \*\* $P < 0.01$ ). Compared to the sham group, CALHM1 expression was significantly increased on the third day after ION-CCI surgery and further increased at 15th day (\* $P < 0.05$ , \*\* $P < 0.01$ ).



**Figure 8**

The changes in protein (CALHM1) expression were confirmed by Western blot. The expression of CALHM1 in the lateral trigeminal nucleus of rats was increased after ION-CCI. The application of ruthenium red did not affect the expression of CALHM1. The expression of CALHM1 was significantly increased in ION-CCI group and NS group compared with control group and sham group ( $n = 6$ , \* $P < 0.05$ , \*\* $P < 0.01$ ). There was no difference in CALHM1 expression between ION-CCI group and NS group compared with RuR group.