

The Role of Pruriceptors in Enhanced Sensitivities to Pruritogens in a Murine Model of Chronic Compression of Dorsal Root Ganglion

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Research

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Abstract

Background: Chronic pruritus is a symptom that commonly observed in neurological diseases. It has been hypothesized that the chronic pruritus may result from sensitization of itch-signaling pathways but the mechanisms remain obscure.

Methods: In this study, we established a mouse model of chronic compression of dorsal root ganglion (CCD) and injected various pruritogenic and algogenic agents intradermally to the calf skin ipsilateral to the compressed DRG. We additionally investigated if pruritogen-evoked activities of dorsal root ganglion (DRG) neurons is enhanced in this model. The expression of TRPV1, CGRP and H1R was detected with immunoflorescent staining. DRG neurons response to four agents using in vivo calcium imaging.

Results: Compared to the naïve mice, a significant increase in itch-related behaviors was observed in the CCD mice after the injection of pruritogens including histamine and BAM8-22, but not after the injection of algogenic agents including capsaicine and 5-HT, although all the above agents evoked enhanced pain-related behaviors toward the injected site. In vivo calcium imaging revealed that compressed DRG neurons exhibited significantly enhanced responses to histamine and BAM8-22. Immunoflorescent staining also showed that the histamine receptor H1 and the capsaicin receptor TRPV1 were significantly upregulated in DRG neurons.

Conclusions: Our findings indicated that sensitization of primary pruriceptive neurons may underlie the enhanced itch sensation after chronic compression of DRG neurons in mice, and may play a role in chronic pruritus in neurological diseases.

Background

Itching (pruritus) has been defined as an “unpleasant skin sensation that elicits the desire or reflex to scratch” [1]. Primary sensory neurons in dorsal root ganglia (DRG) play an important role in generating itch by detecting pruritogenic stimuli via their peripheral axons in the skin and sending the signals to the spinal cord through their central axons [2]. Although both somatosensory sensations activating sensory nerves, itch and pain can be differentiated by psychophysiological and molecular characteristics [3]. Compression of the cervical spinal cord or the spinal ganglia at C5/C6 occurs in brachioradial pruritus (BRP), causing unilateral or bilateral pruritus in the forearms [4].

Chronic compression of the dorsal root ganglion (CCD) is an animal model of lumbar intraforaminal stenosis and radicular pain [5, 6]. CCD increased the incidence of paw-shaking to a normally suprathreshold nociceptive force (“hyperalgesia”) [7, 8]. Hypersensitivity of mechanical behavior may arise in the rat after CCD or after the local application of either proinflammatory tissue to the lumbar DRG [9-11]. The cell bodies of sensory neurons of the compressed DRG become hyperexcitable as evidenced by the presence of spontaneous activity originating in the DRG [12, 13], raised responses to electrical, thermal and chemical nociceptive stimuli [14]. Although the pathophysiology of low back pain is well studied, the neural mechanisms accompanying itch are not largely explored.

Histamine is released from mast cells when tissues are inflamed or stimulated by allergens, and once released, histamine induces itch is triggered by the excitation of a subset of unmyelinated C-fibers [15].

BAM8-22 was agonist which can bind to and activate hMrgX1, mMrgC11 and rMrgC receptors with nanomolar affinities[16-18]. The study of BAM8-22 is of particular interest not only because of its important role in pain transmission and modulation, but also for its highest metabolic stability and longest duration of action compared to the other Mrg neuropeptides agonists[18].

The present study was designed to explore whether there might be an enhanced behavioral responses to pruritogens in the CCD mice and the potential role of primary pruriceptive neurons in mediating the itch-related behaviors.

2. Methods

2.1 Animals

C57BL/6 male mice in all experiments, male GCaMP 3⁺ mice and MrgprA3⁺ mice used in confocal image experiment (Charles River, Wilmington, MA), each weighing 25-30 g were maintained on a 12-hour light/dark cycle. All animal welfare and experimental procedures were in strict according with the Guide for the Care and Use of Laboratory Animals and related ethical regulations of IBMS PUMC, according with the guidelines provided by the International Association for the Study of Pain and National Institutes of Health. Mice were given an ad libitum access to a standard diet and water. Mice were divided into a control group and CCD model group.

2.2. Surgical treatment

Under 3% isoflurane anesthesia, a midline incision was made along the back and the intervertebral foramina of L3 and L4, exposed after separating the paraspinal muscles from the mammillary process and the transverse process [4]. CCD was produced by the insertion of an L-shaped stainless steel rod (0.3 mm diameter, each arm, 2 mm in length), into each foramen to compress DRGs [7].

The incision was closed in layers and topically treated with ointment containing an antibiotic (TriTop), which is a local anesthetic and an anti-inflammatory agent. A systemic antibacterial was also administered (Baytril, 10 mg/mL, i.m.).

After completion of all behavioral testing, mice were euthanized, and DRGs receiving CCD were microscopically examined to confirm rod placement and, after removal of the epineurium and flushing with saline.

2.3. Behavioral test

In this test three groups of mice were given subcutaneous injection of capsaicin (0.1, 1, 10 mg/10 mL), histamine (10, 20, 50 mg/10 mL) and BAM8-22(0.1, 1, 10 mg/10 mL) into the calf of hind leg respectively

and sequent behavior was videotaped using a high definition camera for 30 min on pre-CCD 1d and post-CCD 1, 3, 5, 7d. According to present studies, the injection of capsaicin tends to bring out nociceptive (painful) sensations which lead to licking toward injection site in calf models while pruritic stimulus generally aroused biting behaviors. Hence cumulative durations of licking and biting the injection site were counted through video, taking as the assessment of chemical-induced pain and itch.

The chamber was specially made from a cylindrical glass container (20cm, diameter) with two small mirrors attached to plastic bricks and placed as right angle inside, offering a wide view on every act of the animal. There were 10 min of habituation before each test and recording started immediately after the injection.

2.4. DRG exposure surgery for in vivo imaging of the whole L4 DRG

For all imaging experiments, mice 8 weeks or older were anesthetized by injection of sodium pentobarbital (40-50 mg/kg, i.p.). After deep anesthesia was reached, the animal's back was shaved and aseptically prepared, and ophthalmic ointment was applied to the eyes to prevent drying. During the surgery, mice were kept on a heating pad (DC temperature controller, FHC) to maintain body temperature at 37 ± 0.5 °C as monitored by a rectal probe.

Dorsal laminectomy in DRG was performed usually at spinal level L5 to L3 below the lumbar enlargement but without removing the dura. A 1.5 cm long midline incision was made around the lower part of the lumbar enlargement area; these were dissected away to expose the lower lumbar part which surrounds (L3-L5) vertebra bones. The L4 DRG transverse processes were exposed and cleaned. Using small rongeurs, the surface aspect of the L4 DRG transverse process near the vertebra was removed (only the L4 DRG transverse process was removed but the bone over the spinal cord was intact) to expose the underlying DRG without damaging the DRG and spinal cord. Bleeding from the bone was stopped using styptic cotton.

2.5. In vivo L4 DRG calcium imaging

In vivo imaging of whole L4 DRG in live mice was performed for 5 days after CCD surgery. After surgery mice were laid down in the abdomen down position on a designed microscope stage. The spinal column was stabilized using clamps to minimize movements caused by breathing and heart beats. The mice were maintained under continuous anesthesia for the duration of the imaging experiment with 1-2% isoflurane gas using a gas vaporizer. Pure oxygen air was used to deliver the gas to the mouse.

The microscope stage was fixed under a laser-scanning confocal microscope (Nikon C2 microscope system), which was equipped with macro based large objective and fast EM-CCD camera. Live images were acquired at typically 8-10 frames with 600 Hz in frame-scan mode per 6-7 s, using a 5* 0.5 N.A. macro dry objective, at typically 512*512 pixel resolution with solid diode lasers (Nikon) tuned at 448 wavelength, and emission at 500-550 nm for green fluorescence, respectively. For analysis, raw image stacks (512*512 pixels in the x-y plane; typically 8 optical sections) were imported into Nikon Instrument

system-element for further analysis. DRG neurons were at the focal plane and imaging was monitored during the activation of DRG neuron cell bodies by peripheral chemical stimuli. The imaging parameters were chosen to allow repeated imaging of the same cell over many stimuli, without causing damage to the imaged cells or to surrounding tissue.

2.6. Immunofluorescence

Five days after CCD surgery, the L3 and L4 DRGs of five mice were removed after transcardial perfusion, first with PBS and second, 4% paraformaldehyde, and post-fixed in the same fixative for 4 h, and cryoprotected in 30% sucrose overnight. Tissue was frozen and sectioned at 12 μm thickness by a cryostat and processed for immunofluorescence labeling [19]. The sections on slides were dried at 37°C for 1 hr, and fixed with 4% paraformaldehyde at room temperature for 10 min. The slides were preincubated in blocking solution (10% normal horse serum (vol/vol), 0.2% Triton X-100 (vol/vol) in PBS, pH 7.4) for 1 hr at room temperature, then incubated overnight at 4°C with primary antibodies. Secondary antibody incubation was performed at room temperature for 1 hr.

For primary antibodies, we used rabbit anti-CGRP (T-4239, Peninsula, 1:1,000), rabbit anti-HRH1 (13413-1-AP, 1:400); guinea pig anti-TrpV1 (Abcam, 1:400).

For secondary antibodies, we used Donkey anti-rabbit (A11008, Alexa 488 conjugated; A11011, Alexa 568 conjugated, Thermo Fisher), Donkey anti-guinea pig (706-545-148, Alexa Fluor 680 conjugated; 706-625-148, Alexa Fluor 680 conjugated, Jackson lab). All secondary antibodies were diluted 1:500 in blocking solution. Following washes with PBS, the stained sections were mounted and cover-slipped with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA). The sections were examined and immunostaining images were obtained with an Olympus microscope.

2.7. Quantitative Realtime-RT-PCR

The mRNA levels of TRPV1, Histamine receptor 1, Histamine receptor 4, MrgprA3 receptor in the DRG were measured by real-time PCR (RT-qPCR). Total RNA was extracted by using Trizol reagent according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of total RNA by PrimeScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time). Each cDNA sample was amplified for the gene of interest and GAPDH in a 25 μL reaction volume using SYBR1 Premix Ex TaqTM II (Tli RNaseH Plus). All primers used are listed in Table 1. The realtime RT-PCR conditions were 94 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 55 °C for 30 s and 72 °C for 60 s. The mRNA levels of all genes were normalized to GAPDH.

2.8. Statistical analyses

For in vivo experiments, the animals were distributed into various treated groups randomly. All of the results are given as means \pm SEM. Data distribution was assumed to be normal but this was not formally tested. The data were statistically analyzed with two-tailed, paired/unpaired Student's t test and a one-

way or two-way ANOVA. When ANOVA showed significant difference, pairwise comparisons between means were tested by the post hoc Tukey method (SigmaStat, San Jose, CA).

3. Results

3.1. Sensitized DRG neurons enhanced response to chemical of stimuli after CCD

To explore the behavioral effects of TRPV1, Histamine, 5-HT and MrgprA3 receptor after CCD, we used the calf model that allows the differentiation of side-directed itch- and pain-like behaviors in response to pruritic and algescic chemical stimuli [41]. Mice with high dose of capsaicin injection displayed less site-directed spontaneous biting behaviors than mice with low dose of capsaicin injection ($P < 0.05$, $1 \mu\text{g}/10 \mu\text{L}$ vs $0.1 \mu\text{g}/10 \mu\text{L}$; $P < 0.01$, $10 \mu\text{g}/10 \mu\text{L}$ vs $0.1 \mu\text{g}/10 \mu\text{L}$) [Fig. 1A]. Mice with high dose of capsaicin injection displayed less site-directed spontaneous licking behaviors than mice with low dose of capsaicin injection ($P < 0.05$, $1 \mu\text{g}/10 \mu\text{L}$ vs $0.1 \mu\text{g}/10 \mu\text{L}$; $P < 0.01$, $10 \mu\text{g}/10 \mu\text{L}$ vs $0.1 \mu\text{g}/10 \mu\text{L}$) [Fig. 1E]. Intradermal (i.d.) injection of capsaicin ($1 \mu\text{g}/10 \mu\text{L}$) into the calf of CCD mice significantly increased the number of licking bouts but not the number of biting as compared to mice before CCD (Fig.1E). In contrast, injection of capsaicin to CCD mice did not significantly change the number of licking and biting as compared to control mice (Fig.1A). These results indicate that capsaicin elicits more pain than itch within receptive field of DRG neurons after CCD. These findings suggest that capsaicin acts on TRPV1 to trigger pain behaviors in vivo under the CCD conditions.

At 5 days after CCD surgery, mice with histamine injection displayed more site-directed spontaneous biting behaviors than control mice [Fig. 1B]. Mice with high dose of histamine injection displayed more site-directed spontaneous biting behaviors than mice with low dose of histamine injection ($P < 0.01$, $20 \mu\text{g}/10 \mu\text{L}$ and $50 \mu\text{g}/10 \mu\text{L}$ vs $10 \mu\text{g}/10 \mu\text{L}$) [Fig. 1B]. Intradermal (i.d.) injection of histamine ($20 \mu\text{g}/10 \mu\text{L}$) into the calf of CCD mice significantly increased the number of biting bouts but not the number of licking as compared to control mice (Fig.1B, D). These results suggest that histamine elicits more itch than pain within receptive field of DRG neurons after CCD. These findings indicate that histamine acts on histamine receptor to trigger itch behaviors in vivo under the CCD conditions.

We then tested histamine-independent pruritogen, BAM8-22 on evoking skin itch. At 5 days after CCD surgery, mice with BAM injection displayed more site-directed spontaneous biting behaviors than control mice [Fig. 1C]. Intradermal (i.d.) injection of BAM ($1 \mu\text{g}/10 \mu\text{L}$) into the calf of CCD mice significantly increased the number of biting bouts but not the number of licking as compared to control mice (Fig.1C, G). Intradermal (i.d.) injection of BAM ($10 \mu\text{g}/10 \mu\text{L}$) into the calf of control mice significantly increased the number of licking as compared to low dose BAM injection to control mice [Fig.1G]. These results suggest that BAM elicits more itch than pain within receptive field of DRG neurons after CCD.

Mice with 5-HT ($0.003 \mu\text{g}/10 \mu\text{L}$) injection displayed no more site-directed spontaneous biting and licking behaviors between CCD mice and control mice [Fig. 1D, H]. At 1 day after CCD surgery, intradermal (i.d.) injection of 5-HT ($0.3 \mu\text{g}/10 \mu\text{L}$) into the calf of CCD mice significantly increased the number of biting bouts and licking behaviors as compared to control mice (Fig.1D, H). These results suggest that high

dose of 5-HT elicits more itch and pain within receptive field of DRG neurons between control mice and CCD mice.

To compare DRG neurons respond to chemical stimuli between control and CCD, we found the most suitable concentration of chemical. In low and high concentration, there are no significantly different at behavior results between control and CCD. Compared with control group, the calf licking of CCD group increased significantly by using medium concentration chemical (Capsaicin 1 $\mu\text{g}/10 \mu\text{L}$, Histamine 20 $\mu\text{g}/10 \mu\text{L}$ and BAM 1 $\mu\text{g}/10 \mu\text{L}$) (Fig.2A). 0.3 $\mu\text{g}/10 \mu\text{L}$ of 5-HT injection increased the calf licking time in CCD group compared with control group (Fig. 2A). Histamine and BAM increased the calf biting time in CCD group compared with control group (Fig.2B).

3.2. Confocal imaging of DRG

To evaluate neuronal activity in DRG somata, we used Pirt-GCaMP3 mice to image Ca^{2+} response in L4 DRG.

The percentage of neurons that responded to capsaicin (1 $\mu\text{g}/10 \mu\text{L}$) was significantly increased in CCD (169/382, n=6) compared with the control DRG (75/398, n=4), $P < 0.01$, as shown Fig 3A-D, M. The percentage of neurons that responded to histamine (20 $\mu\text{g}/10 \mu\text{L}$) was significantly increased in CCD (149/385, n=4) compared with the control DRG (92/440, n=5), $P < 0.01$, as shown Fig 3E-H, N.

The percentage of neurons that responded to BAM8-22 (1 $\mu\text{g}/10 \mu\text{L}$) was significantly increased in CCD (79/352, n=3) compared with the control DRG (60/362, n=3), $P < 0.01$, as shown Fig 3I-L, O.

In the present study, a model of CCD was used to mimic a chronic neuropathic state. Bilateral L4 DRG of mice were kept in ACSF at 37°C on 5 days after CCD. Using confocal image, we investigated MrgprA3⁺ neuronal activity in L4 DRG somata in vitro. The total number of MrgprA3⁺ neurons was significantly increased in CCD (230, 38.33 \pm 3.07, n=6) compared with the control DRG (133, 18.83 \pm 1.7, n=6), $P < 0.01$, as shown Fig 4A-B.

3.3 H1R and TRPV1 of DRG neurons immunoreactivity after CCD surgery

Histamine receptor 1 immunoreactivity was detected in 10.60% (42/396, n=6) of DRG sensory neurons in control. However, Histamine receptor 1 immunoreactivity was detected in 16.15% (62/384, n=6) in CCD, as shown in Fig 5A. H1R⁺ DRG sensory neurons were of small diameter (average 30 μm), positive for marker TRPV1⁺. TRPV1 immunoreactivity was detected in 12.82% (155/1209, n=10) of DRG sensory neurons in control. However, TRPV1 immunoreactivity was detected in 18.55% (218/1175, n=6) in CCD, as shown in Fig 5B. TRPV1⁺ DRG sensory neurons were of small diameter (average 30 μm), positive for marker CGRP. Immunofluorescent staining revealed very few H1- and TRPV1-immunopositive DRG neurons in control mice (Fig. 6A-C). In contrast, the mean percentage of H1- and TRPV1-immunopositive DRG neurons was significantly greater in CCD mice (Fig. 6E-G). In addition, some H1-immunopositive DRG neurons in CCD mice were also significantly increased immunopositive for TRPV1 (detected in

44.14% of neurons with H1-immunopositive, Fig. 6H) compared with control mice (detected in 26.31% of neurons with H1-immunopositive, Fig. 6D).

Immunofluorescent staining revealed very few TRPV1- and CGRP-immunopositive DRG neurons in control mice (Fig. 7A- C). In contrast, the mean percentage of H1- and TRPV1-immunopositive DRG neurons was significantly greater in CCD mice (Fig. 7D-F). In addition, some TRPV1-immunopositive DRG neurons in CCD mice were also significantly increased immunopositive for TRPV1 (detected in 40% of neurons with CGRP-immunopositive, Fig. 7H) compared with control mice (detected in 21.88% of neurons with CGRP-immunopositive, Fig. 7G).

3.4 Expression of TRPV1/Histamine Receptor on mouse DRG

TRPV1 is expressed in primary sensory neurons in the DRG and is distributed in small-diameter, nociceptive neurons. Because TRPV1 may contribute to production of itch sensation at the primary sensory neurons similar to the function of histamine receptor, we investigated the potential interaction of the two receptors. In our immunofluorescence staining study, immunoactivity for TRPV1 and that for H1R in DRG neurons of CCD mice increased compared with control mice.

It is possible that the sensitization of DRG we detected after CCD is mediated by upregulated and spontaneously released Histamine via activation of HR1A on DRG.

At the protein level, immunofluorescence results revealed that a significantly larger percentage of DRG neurons of CCD mice stained TRPV1 compared with controls (Fig.3A, E), indicating an increased number of cutaneous sensory neurons expressing TRPV1 after the development of CCD (12.82%:155/1209 vs 18.55%:218/1175).

CGRP receptors are expressed on the nervous system. Both peripheral and central neurons released CGRP that play role in inducing central sensitisation to tactile stimuli.

We further determined the expression pattern of H1R in DRG after CCD. The percentage of DRG neurons stained with H1R from CCD mice (16.15%, 62/384) was significantly greater as compared with that from control mice (12.17%, 50/411).

3.5 CCD elevated mRNA expression of TRPV1 and MrgprA3 in CCD DRG

As shown in Fig.8, mean mRNA expression levels of TRPV1, Histamine receptor 1 and MrgprA3 measured in the DRG, were significantly elevated in the CCD model group ($P < 0.05$) compared to control.

4. Discussion

In this study, we first found that CCD upregulated H1R and TRPV1 coexpression in primary sensory neurons of dorsal root ganglion. The injection of low dose of histamine and BAM into the calf of mice

evoked itch-like behaviors after CCD. There were a significantly greater percentage of primary sensory neurons expressing not only H1R but also TRPV1 after CCD.

Researcher found that both H1R and H4R are expressed on C-afferent fiber terminals, and also that these antagonists can directly inhibit the transmission of itching responses from the peripheral to central nervous system [20]. Using single-cell calcium imaging, Rossbach *et al.* found that histamine induced an increase in calcium levels in a subset of skin-specific sensory neurons in mice by activating H1R and H4R as well as inhibiting H3R [21].,

Histamine is a well-known mediator of acute inflammatory and immediate hypersensitivity responses. Though the physiological role of histamine was well studied, much is known of signaling pathway that leads to the excitation of the sensory neurons, which induces the adapt neural signals for itching. Our study provides in vivo and in vitro evidence that histamine requires the activation of TRPV1 to excite primary sensory neurons of DRG after CCD. H1R can activate phospholipase C, and increases intracellular Ca^{2+} level. Pruritus is elicited by the activation of H1R.

Transient receptor potential cation channel, subfamily V, member 1 (TRPV1) is an important molecular component of pain detection and modulation at peripheral and central nociceptive neurons [22, 23]. Moreover, research showed that histamine induces itch by activating PLA2, lipoxygenase, and the TRPV1 signaling pathway [24]. Histamine induces inward currents that are blocked by antagonists of TRPV1 [24] [25].

The strong relationship between histamine and TRPV1 in primary sensory neurons of DRG has been showed in our study. Coexpression of TRPV1 and histamine receptor is in a subset of sensory neurons [26, 27], and primary afferent C-fibers that respond to histamine are also sensitive to capsaicin [26, 28]. Moreover, repetitive application of capsaicin is known to desensitize TRPV1 or sensory nerves and was found to alleviate the pruritus induced by histamine [29]. In a word, these results further strengthen the notion that TRPV1 mediates histamine-induced itching. There are some reports suggest that histamine H1 and H4 receptors are co-involved in the pathway to transmit the itch signal to the central system [30, 31]. In the present study, we showed that CCD increased histamine H1 and TRPV1 receptor agonist-induced itching behaviors.

After the histamine H1 receptor was activated, the Gαq proteins coupled with the histamine H1 receptor downstream signal pathway induced TRPV1 to open and excited the neurons to transmit the itch signal [24, 25].

BAM8-22 was fragment from the proenkephalin A gene, which was identified as ligand capable of potently activating rat MrgC11 and MrgA3. After CCD, the expression of MrgA3 protein in DRG elevated compared to control mice.

In this study, mRNA of DRG tissue was detected, single cell PCR should be applied to detect mRNA expression of histamine receptor and TRPV1 positive neuron in next study. Undoubtedly, more work is

needed to understand how these **pruriceptors** enhances **pruriceptic** behaviors in mouse.

5. Conclusions

In summary, the present study shows that lower concentration of histamine and BAM excite sensory neurons to induce itching behavior in CCD mice, not in control mice.

The responses of compressed DRG neurons to histamine and BAM8-22 were significantly enhanced, and its H1R and TRPV1 were markedly upregulated. Moreover, histamine is an important cause of itching in dermatitis patient, the present study provides clues concerning the treatment of evoked-itching and inflammatory pain.

Abbreviations

CCD: Chronic compression of dorsal root ganglion; DRG: dorsal root ganglion; CGRP: Calcitonin gene-related peptide; TRPV1: Transient receptor potential vanilloid-1; BAM: Bovine adrenalmedulla8-22; 5-HT: 5-hydroxytryptamine; H1R: Histamine receptor 1.

Declarations

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Ethics approval:

All animal procedures performed in this study were reviewed and approved by the Institutional Animal Care and Use Committee in Chinese Academy of Medical Sciences, Institute of Basic Medical Sciences and were conducted in accordance with the guidelines of the International Association for the Study of Pain.

Availability of data and materials: There is no data, software, databases, and application/tool available apart from the reported in the present study. All data is provided in manuscript and supplementary data.

Author contributions:

JT collected the behavioral data and performed DRG calcium imaging, TW performed animal surgery, Immunofluorescence and Realtime PCR, and the statistical analyses. TW and JT drafted the manuscript. CM supervised the project and revised the manuscript.

Competing interests:

All authors declare no conflicts of interest.

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Table

Table 1 The sequence of the primers used in the experiment.

Gene	Primer sequence (5'-3')
Trpv1-F	CCGGCTTTTTGGGAAGGGT
Trpv1-R	GAGACAGGTAGGTCCATCCAC
Hrh1-F	CAAGATGTGTGAGGGGAACAG
Hrh1-R	CTACCGACAGGCTGACAATGT
Hrh4-F	GTCCCCTTGGCATTTTTAATGTC
Hrh4-R	ACATGCAGATTCCACTTCCAAA
Mrgpra3-F	CTCAAGTTTACCCTACCCAAAGG
Mrgpra3-R	CCGCAGAAATAACCATCCAGAA

Figures

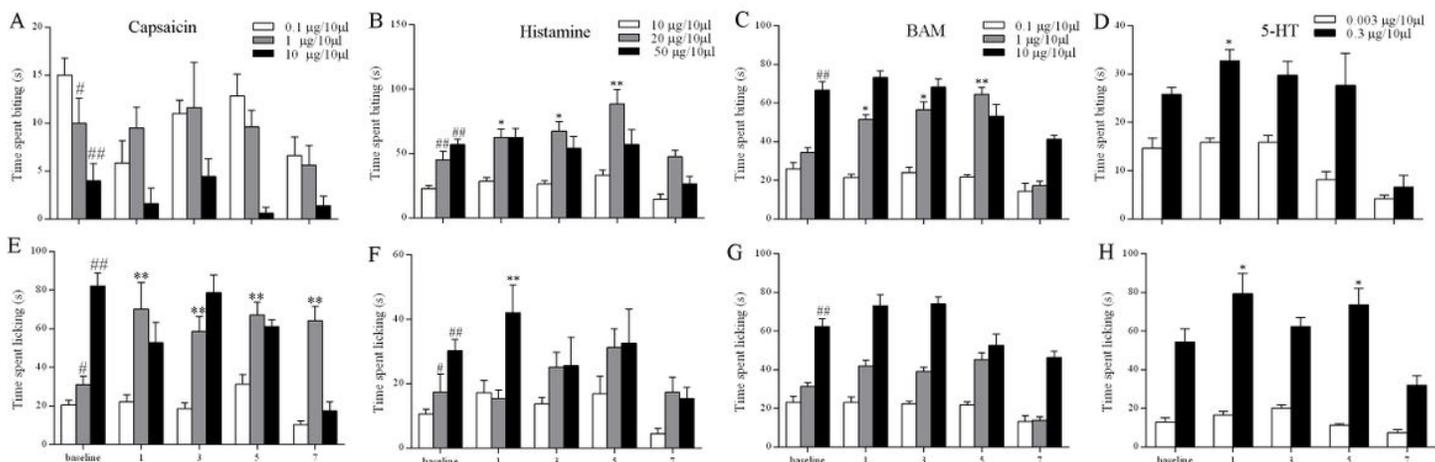


Figure 1

Capsaicin, Histamine, BAM and 5-HT induce itch-like and pain-like behavior in the calf injection model. A, E) Total time spent biting or licking on the calf over the 30 min observation period immediately after

injection of capsaicin (0.1 μ g/ 10 μ L, 1 μ g/ 10 μ L and 10 μ g/ 10 μ L). B, F) Total time spent biting or licking on the calf over the 30 min observation period immediately after injection of Histamine (10 μ g/ 10 μ L, 20 μ g/ 10 μ L and 30 μ g/ 10 μ L). C, G) Total time spent biting or licking on the calf over the 30 min observation period immediately after injection of BAM8-22 (0.1 μ g/ 10 μ L, 1 μ g/ 10 μ L and 10 μ g/ 10 μ L). D, H) Total time spent biting or licking on the calf over the 30 min observation period immediately after injection of 5-HT (0.003 μ g/ 10 μ L and 0.3 μ g/ 10 μ L). Data are mean \pm SEM of 6 mice per group. *P<0.05, **P<0.01; #P<0.05, ##P<0.01, Student's t test.

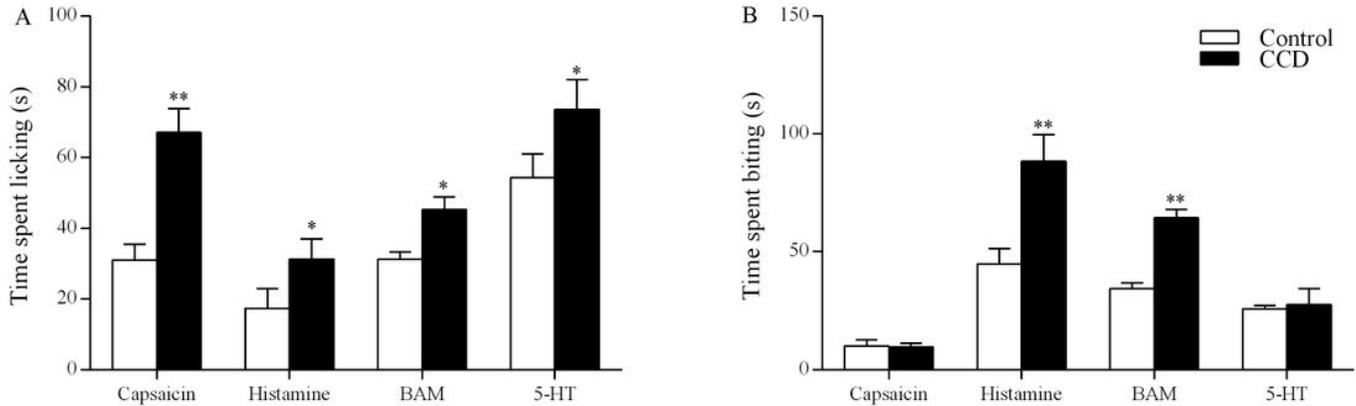
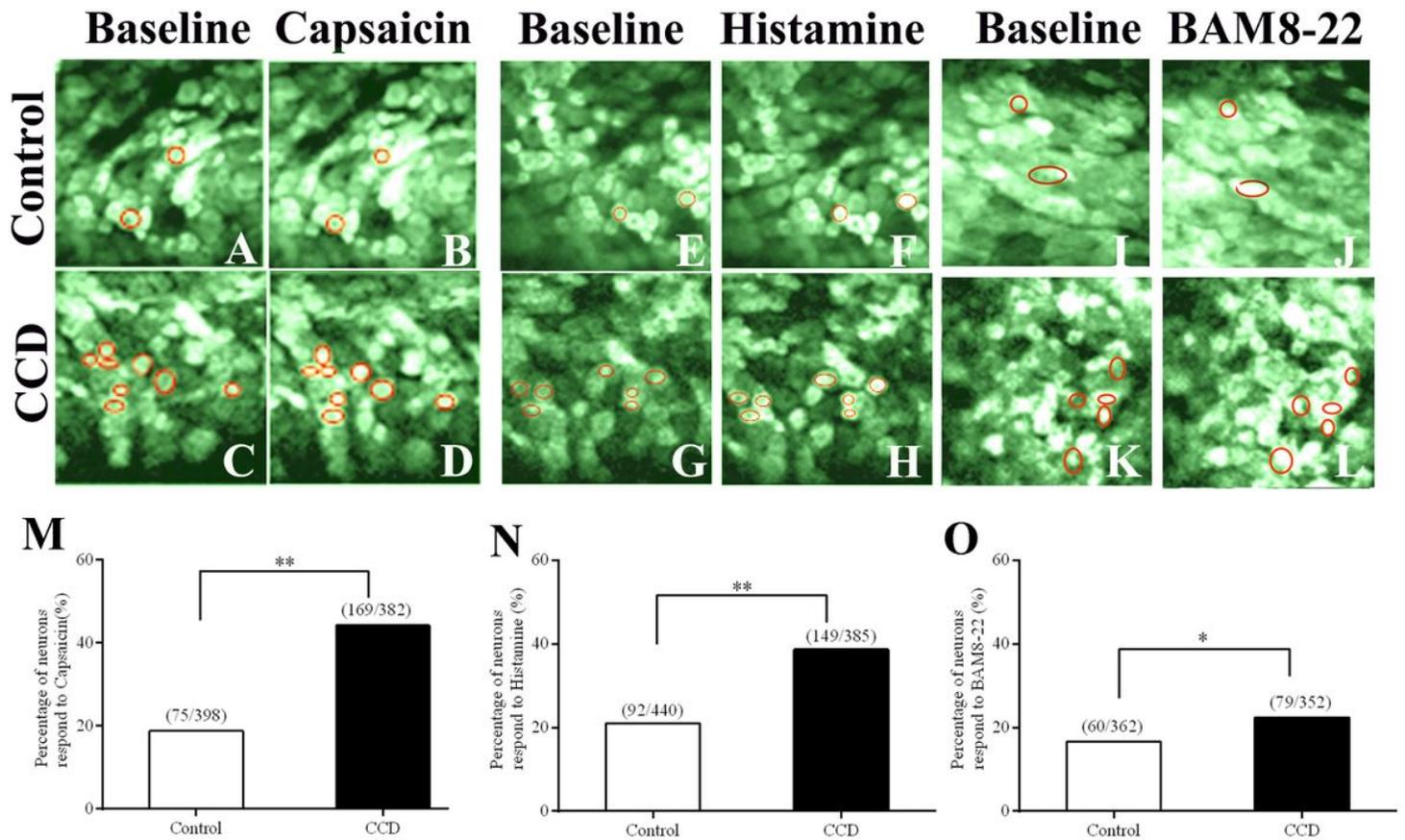


Figure 3

Intradermal chemical (capsaicin, histamine, BAM and 5-HT) injection in the calf of mice induced licking and biting, which is increased at 5 days after chronic compression of dorsal root ganglion. A) Total time spent licking on the calf over the 30 min observation period immediately after injection of capsaicin (1 μ g/ 10 μ L), histamine (20 μ g/ 10 μ L), BAM (1 μ g/ 10 μ L), and 5-HT (0.3 μ g/ 10 μ L). B) Total time spent biting on the calf over the 30 min observation period immediately after injection of capsaicin (1 μ g/ 10 μ L), histamine (20 μ g/ 10 μ L), BAM (1 μ g/ 10 μ L), and 5-HT (0.3 μ g/ 10 μ L). *P<0.05, **P<0.01, CCD vs Control.



Calcium imaging of activity in L4 DRG neurons after CCD in pirt-GCaMP3s mice. (A-D) Representative in vivo images of neurons labeled for GFP, and activated after intradermal injection of capsaicin. (M) Percentage of neurons respond to capsaicin (1 μ g/ 10 μ L) to control and CCD mice, ** $P < 0.01$. (E-H) Representative in vivo images of neurons labeled for GFP, and activated after intradermal injection of histamine. (N) Percentage of neurons respond to histamine (20 μ g/ 10 μ L) to control and CCD mice, ** $P < 0.01$. (I-L) Representative in vivo images of neurons labeled for GFP, and activated after intradermal injection of BAM8-22. (O) Percentage of neurons respond to BAM8-22 (1 μ g/ 10 μ L) to control and CCD mice, * $P < 0.05$.

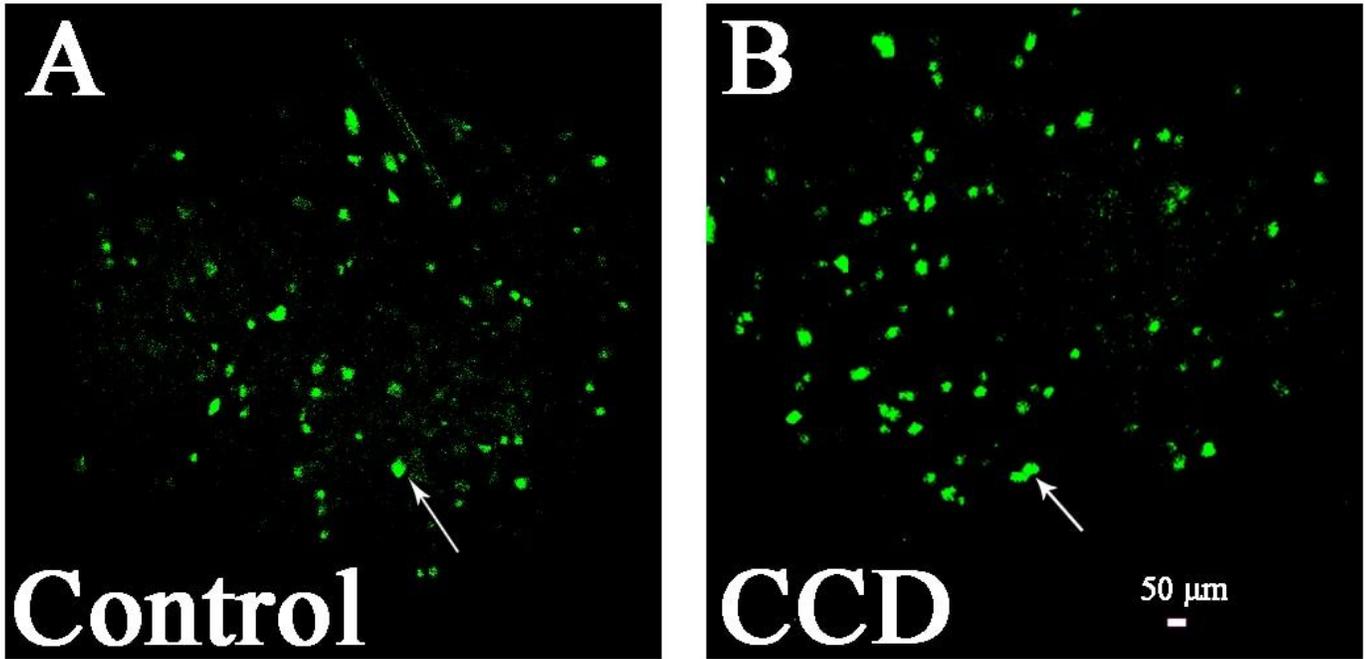


Figure 7

Upregulation of MrgprA3⁺ sensory neurons of L4 dorsal root ganglion in CCD mice. A, Control mice; B, CCD mice. Confocal image showed an increased the total number of neurons in L4 DRG neurons in CCD mice compared with that in control mice (n=6 mice/group). *P<0.05, CCD vs Control, Student's t-test. Scale bar indicates 50 μm.

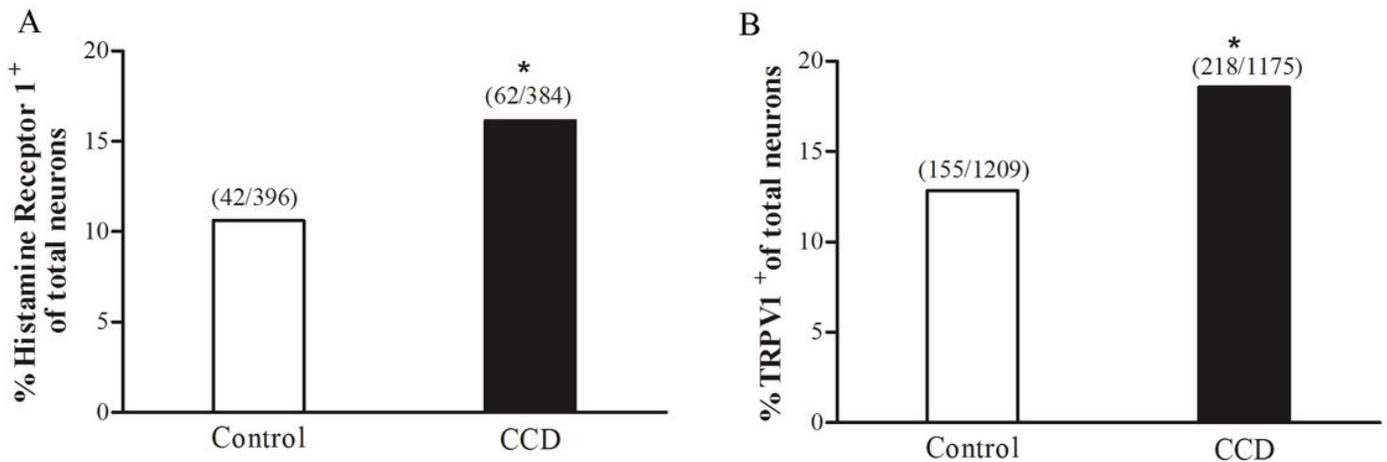


Figure 9

Upregulation of the expression of H1R and TRPV1 in the DRG in CCD mice. A and B. Immunofluorescence staining showed a significantly higher percentage of H1R- and TRPV1-immunopositive DRG neurons in CCD mice compared with that in control mice (n=6 mice/group). *P<0.05, CCD vs Control, Student's t-test.

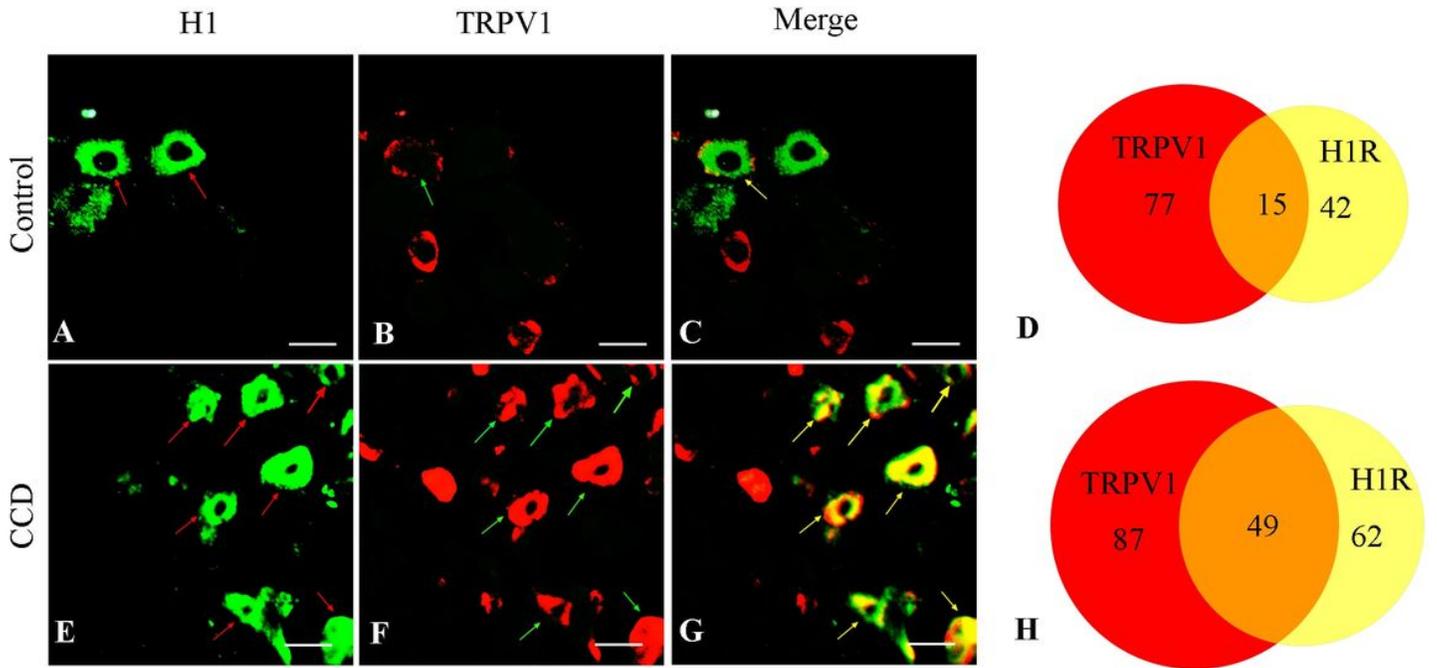


Figure 11

Percentage of DRG neurons that express H1 and TRPV1 in CCD mice. A-B and E-F. Typical microscopic images of immunofluorescence staining for H1 (A and E) and TRPV1 (B and F) from Control and CCD mice. C and G. H1 co-expressed with TRPV1 in some DRG neurons in CCD mice. D and H. Quantification of H1 and TRPV1 coexpression in the DRG neurons between Control and CCD mice. Red, green and yellow arrows point toward H1+, TRPV1+ and merge neurons, respectively. Scale bars indicate 25 μ m.

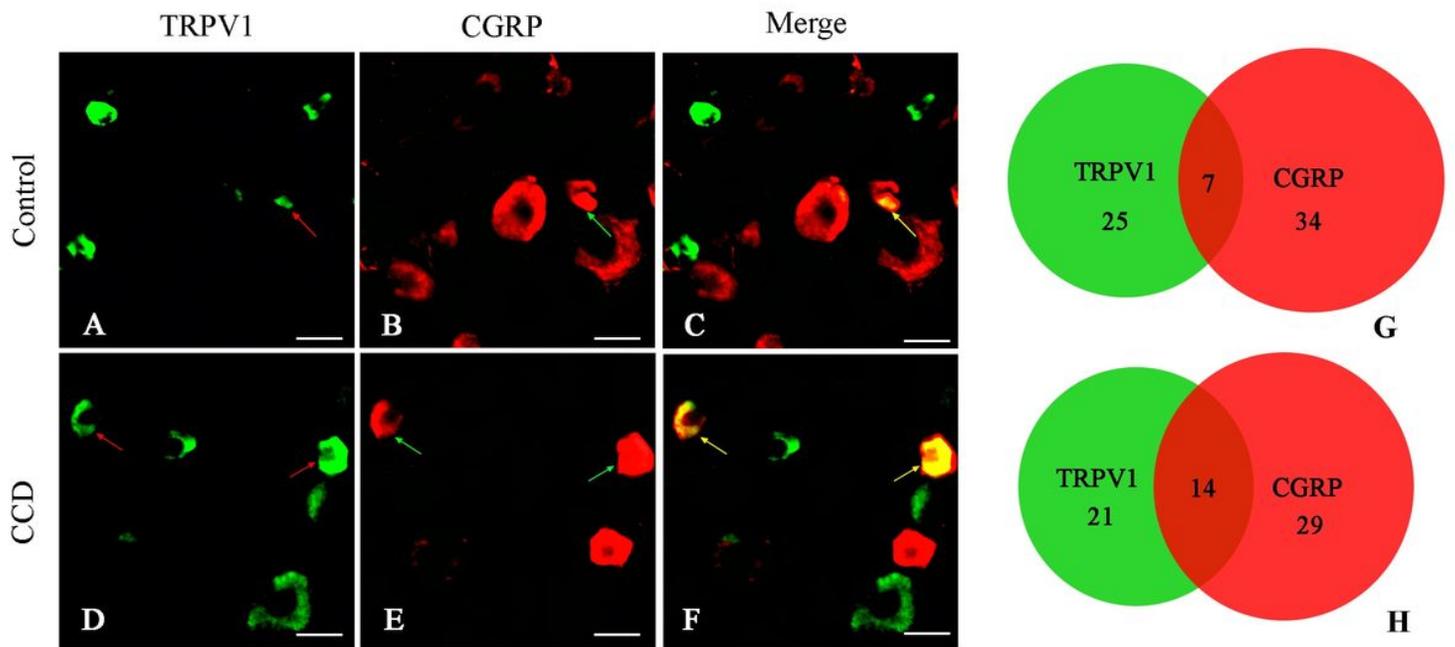


Figure 13

Percentage of DRG neurons that express TRPV1 and CGRP in CCD mice. A-B and E-F. Typical microscopic images of immunofluorescence staining for TRPV1 (A and D) and CGRP (B and E) from Control and CCD mice. C and F. TRPV1 co-expressed with CGRP in some DRG neurons in CCD mice. G and H. Quantification of TRPV1 and CGRP coexpression in the DRG neurons between Control and CCD mice. Red, green and yellow arrows point toward TRPV1+, CGRP+ and merge neurons, respectively. Scale bars indicate 25 μ m.

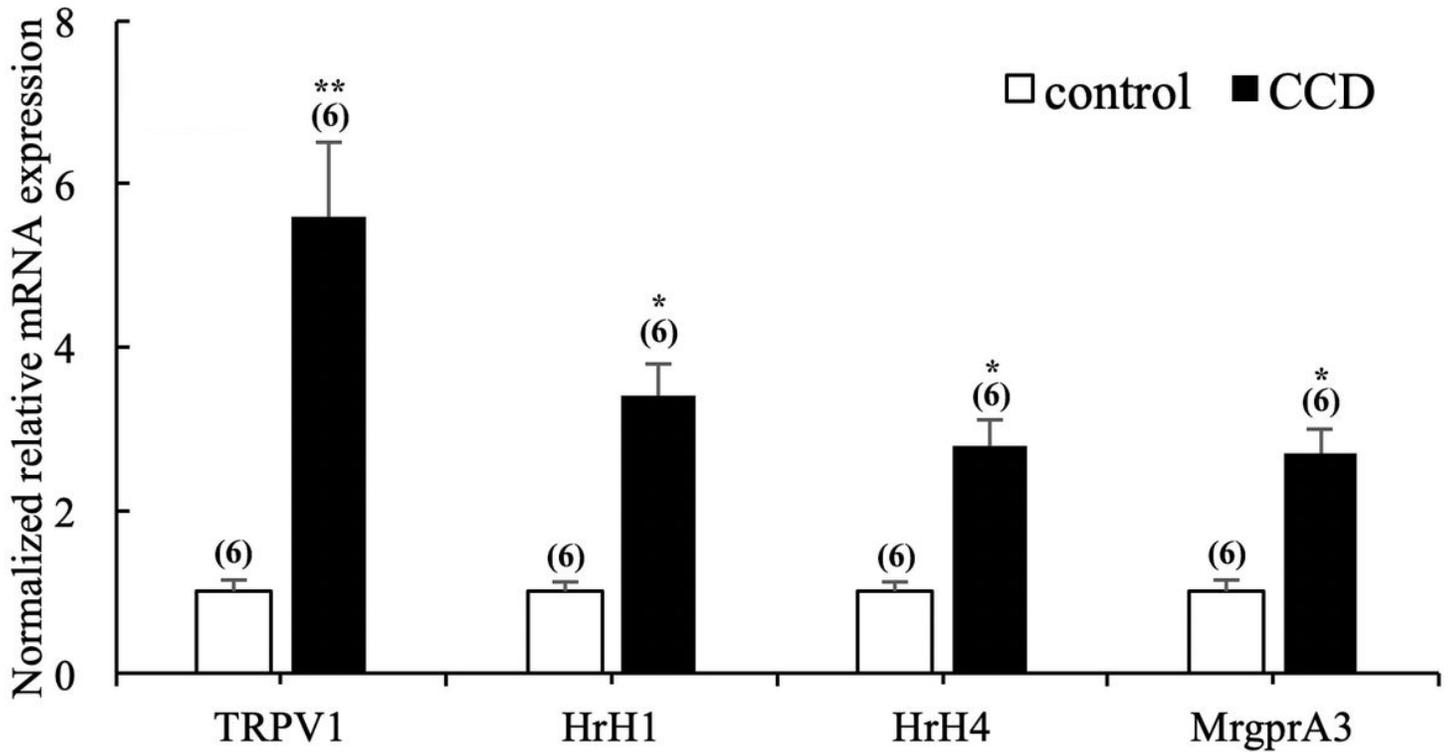


Figure 15

Upregulation of mRNA expression of TRPV1, HrH1, HrH4 and MrgprA3 in the DRG in CCD mice. Realtime RT-PCR showed a significant increase in mRNA expression levels of TRPV1, HrH1, HrH4 and MrgprA3 in L3-L4 DRG for CCD vs Control mice 5 days after the surgery (n=6 mice/group for CCD, n=6 mice/group for Control).