

TRPA1 Ion Channel Mediates the Analgesic Effects of Acupuncture at the ST36 Acupoint in Mice Suffering from Arthritis

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Purpose: Acupuncture (ACU) has been demonstrated to alleviate inflammatory pain. Mechanoreceptors are present in acupuncture points. When acupuncture exerts mechanical force, these ion channels open and convert the mechanical signals into biochemical signals. TRPA1 (Transient receptor potential ankyrin 1) is capable of sensing various physical and chemical stimuli and serves as a sensor for inflammation and pain. This protein is expressed in immune cells and contributes to local defense mechanisms during early tissue damage and inflammation. In this study, we investigated the role of TRPA1 in acupuncture analgesia.

Patients and Methods: We injected complete Freund's adjuvant (CFA) into the mouse plantars to establish a hyperalgesia model. Immunohistochemistry and immunofluorescence analyses were performed to determine the effect of acupuncture on the TRPA1 expression in the Zusanli (ST36). We used TRPA1^{-/-} mouse and pharmacological methods to antagonize TRPA1 to observe the effect on acupuncture analgesia. On this basis, collagenase was used to destroy collagen fibers at ST36 to observe the effect on TRPA1.

Results: We found that the ACU group vs the CFA group, the number of TRPA1-positive mast cells, macrophages, and fibroblasts at the ST36 increased significantly. In CFA-inflammatory pain models, the TRPA1^{-/-} ACU vs TRPA1^{+/+} ACU groups, the paw withdrawal latency (PWL) and paw withdrawal threshold (PWT) downregulated significantly. In the ACU + high-, ACU + medium-, ACU + low-dose HC-030031 vs ACU groups, the PWL and PWT were downregulated, and in carrageenan-induced inflammatory pain models were consistent with these results. We further found the ACU + collagenase vs ACU groups, the numbers of TRPA1-positive mast cells, macrophages, and fibroblasts at the ST36 were downregulated.

Conclusion: These findings together imply that TRPA1 plays a significant role in the analgesic effects produced via acupuncture at the ST36. This provides new evidence for acupuncture treatment of painful diseases.

Keywords: acupuncture, TRPA1, ST36, inflammation, immune, analgesia

Introduction

Inflammatory pain is caused via the activation of various mediators released at the site of inflammation in tissues, which is manifested as a decrease in the pain threshold and an increase in the sensory neuron reactivity.¹ A moderate inflammatory response in the early stage of the disease exhibits a protective and defensive effect and can restore the homeostasis of the internal environment of the body. However, long-term disordered and uncontrolled inflammation reactions can damage cells and tissues. Inflammatory mediators, which are key to initiating and maintaining pain,² include proinflammatory cytokines,

reactive oxygen species (ROS), adenosine triphosphate (ATP) and other factors. After a tissue injury, these mediators act via the infiltration of leukocytes, vascular endothelial cells, or hypertrophic cell release. Mediators related to inflammatory responses directly or indirectly activate transient receptor potential (TRP) channels on the nociceptors of peripheral neurons, thereby inducing neurogenic inflammatory responses, transmitting pain signals to primary sensory centers, and activating neurons to release substance P (SP) and calcitonin gene-related peptide (CGRP) in order to transmit pain.³ Therefore, sensitization of the TRP channels in sensory nerves during inflammation can be a major mechanism of inflammatory pain.

Acupoint activation is a process wherein acupuncture points receive physical stimulation, which is then converted into biochemical signals.⁴ This process involves the interaction of local microcirculation, connective tissue, and immune cells. A mechanical coupling occurs between the subcutaneous collagen fibers and the needle body during acupuncture, and the collagen fibers interweave and scatter to form a spatial network structure in the connective tissues. Tissues surround the needle, and this signal is transmitted to the mast cells, leading to their degranulation.⁵ Ding et al reported that the local mechanosensitive channel (MSc) in the acupoint area is connected to the surrounding collagen fibers.⁶ The MSc receives mechanical signals of acupuncture transmitted from the collagen fibers, and the collagen fibers participate in the transmission of acupuncture signals from the acupoints to the target organs and the conversion process. Previous studies have demonstrated that mechanical stimulation by acupuncture can also contract fibroblasts, pulling in more collagen, causing matrix deformation, and lastly transmitting force signals via connective tissues.^{7–9} Zhang et al used flow cytometry to detect macrophages at the ST36 in a CFA-induced rat model and reported that skin macrophages increased significantly after acupuncture, which confirms that acupuncture can induce key immune cells and macrophages related to acupuncture points.¹⁰

TRP channels can integrate and convert mechanical stimuli into chemical signals. Previous studies have displayed that TRP channels, which are detectors of noxious stimuli, are transducers of exogenous and endogenous noxious signals, regulate the excitability of neurons in pain and inflammatory states, and can be targeted for pain relief.^{11–13} They open upon activation via noxious stimuli. The membrane potentials are changed, action potentials are generated, and signals from the peripheral nervous system (PNS) are transmitted to the spinal cord.¹⁴ TRPA1 is one of the members of the TRP family.¹⁵ TRPA1 involved in vital functions such as body temperature, taste, hearing, and osmotic perception.¹⁶ Recent studies have reported that TRPA1 is activated via the release of inflammatory factors from non-neuronal cells in areas of tissue injury or disease. Local TRPA1 activators trigger the skin neuroimmune regeneration cascade, thereby promoting tissue regeneration in adult mammals and participating in skin physiological functions,¹⁷ which suggests that TRPA1 is crucial for immune system cells.

Therefore, this study uses acupuncture to treat arthritis mouse inflammatory pain model as a research platform. On the basis of confirming the effectiveness of acupuncture analgesia, we explore the cell types expressing TRPA1 in ST36 and whether TRPA1 is involved in the analgesic effect of acupuncture. And explore whether the triggering factor of TRPA1 is the entanglement of collagen fibers, reveal the key source link in the complex process of acupuncture, and provide new scientific evidence for the activation mechanism of acupuncture acupoint effects. We found that the TRPA1 expression in mast cells, macrophages, and fibroblasts in the acupoints of mice was upregulated after acupuncture. In the inflammatory pain model, the acupuncture analgesic effect of TRPA1 knockout (KO) mice was lower than that in the wild type (WT) mice, confirming that TRPA1 is involved in the analgesic effect of acupuncture in mice, which was further confirmed by injecting TRPA1 antagonists into the ST36. TRPA1 activated the anti-inflammatory and analgesic effect of acupuncture. The collagen fibers of the ST36 were destroyed and the analgesic effect of acupuncture was gradually reduced, such that the mechanical force generated by acupuncture was transmitted to the deep tissues of the acupoint via the collagen fiber. To summarize, our findings suggest that TRPA1 ion channels play a crucial role in initiating inflammatory responses via acupuncture at acupoints and that regulating TRPA1 can be a novel method for treating inflammatory pain.

Materials and Methods

Animals

In this study, specific-pathogen-free (SPF) C57BL/6J adult male WT mice (age: 7–8 weeks, weight: 20–22 g) and KO mice were used. The WT mice were provided by the Beijing Weitong Lihua Experimental Animal Technology. The KO mice were obtained from the Shanghai Jiaotong University Laboratory.¹⁸ All mice were maintained at the Experimental Animal Center of Tianjin University of Traditional Chinese Medicine for breeding and standby. All experimental mice were kept in cages

(4 mice/cage) measuring 3.20 cm long \times 2.10 cm wide \times 1.60 cm high, and sterilized corn-cob bedding was provided in the cage. The mice were maintained in a 12 h day/night photophase (08:00 AM to 08:00 PM, humidity 55–65%). All mice were provided free access to drinking water and standard feed. The experimental procedure strictly followed the Guidelines for the Care and Use of Laboratory Animals and was approved by the Experimental Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (approval number: TCM-LAEC2021279).

Study Design

Experiment 1, the mice were randomly assigned to the control, CFA, and ACU groups ($n = 8/\text{group}$). The mice in the ACU group were treated with ST36 on both sides. The behavioral indicators were measured daily after acupuncture, and the TRPA1 expression and cell types were determined by using immunohistochemistry and immunofluorescence staining techniques.

Experiment 2, we injected CFA into the paws of WT and KO mice to induce a mouse model of inflammatory pain. The mice were randomly assigned to the TRPA1^{+/+} control group, TRPA1^{+/+} model group, TRPA1^{+/+} ACU group, TRPA1^{-/-} control group, TRPA1^{-/-} model group, or TRPA1^{-/-} ACU group ($n = 8\text{--}9/\text{group}$). They were measured daily for their behavioral indicators after acupuncture and then the pain of the two types of mice in the normal, model, and acupunctured states was compared. Afterward, the mice were randomly assigned to the control, CFA, ACU, ACU + high-dose, ACU + medium-dose, and ACU + low-dose groups ($n = 6/\text{group}$). In the antagonist group, 20 μL of TRPA1 channel antagonist HC-030031 of different concentrations was injected into the bilateral ST36 30 min before the acupuncture, and 20 μL of the DMSO solution was injected in the control mice. On the basis of this experiment, mice were injected with carrageenan to create another inflammatory-pain mouse model, and the experimental grouping was consistent with that of the CFA model.

Experiment 3, the mice were randomly assigned to the control, CFA, ACU, and ACU + collagenase groups ($n = 6/\text{group}$). The ACU group was injected with 20 μL of TESCA into the ST36. The ACU + collagenase group received 2.5 mg/mL of collagenase in the ST36 30 min before the acupuncture, and the expression of TRPA1 was observed using immunofluorescence staining. All experiments were performed in a double-blind manner.

Genetic Identification

Genomic DNA was extracted using a small and rapid extraction kit (BOSTER, MK041-3, Wuhan, China). Polymerase chain reaction (PCR) was performed using a reaction system (25 μL) prepared with 2XTaq Master Mix (Novoprotein, E005-02B, Wuhan, China), 12.5 μL , F-Prime, 0.5 μL , R-Prime, 0.5 μL (Sangon, Shanghai, China), DNA, 1 μL , ddH₂O (BOSTER, BL510A, Wuhan, China), 10.5 μL , oLMR8645 gene (F-Prime, CCT CGA ATC GTG GAT CCA CTA GTT CTA GAT, R-Prime, GAG CAT TAC TTA CTA GCA TCC TGC CGT GCC), oLMR9179 gene (F-Prime, TCC TGC AAG GGT GAT TGC GTT GTC TA, R-Prime, TCA TCT GGG CAA CAA TGT CAC CTG GT), target fragment size WT = 317 bp, KO = 184 bp, heterozygote = 184 bp and 317 bp, under the following reaction conditions. Stage 1, pre-denaturation: 94 $^{\circ}\text{C}$, 2 min; 94 $^{\circ}\text{C}$, 20s; 65 $^{\circ}\text{C}$, 15s; 68 $^{\circ}\text{C}$, 10s; 9 cycles. Stage 2: 94 $^{\circ}\text{C}$, 15s; 60 $^{\circ}\text{C}$, 15s; 72 $^{\circ}\text{C}$, 10s; 27 cycles. Stage 3, 72 $^{\circ}\text{C}$, 2 min; 4 $^{\circ}\text{C}$. Genotype comparison analysis was performed via gel imaging using an analysis system (Bio-rad, California, USA).

Inflammatory Pain Model Establishment

All mice, except the control ones, were modeled. The control mice were intradermally injected with 0.9% normal saline (0.05 mL), and the mice in the model and acupuncture groups were injected with CFA (0.05 mL, 1 mg/mL, Sigma, St. Louis, MO, USA). In a KO experiment, a 3% carrageenan solution (0.05 mL, Sigma, St. Louis, MO, USA) was dissolved in normal saline and injected into the plantar surface of the right hind paw of the mouse¹⁹ to induce an inflammatory response on the right side. Development of abnormal redness and swelling on the right sole of the mouse, decreased movement, and lameness indicated that the modeling was successful.

Acupuncture Simulation

We referred to the technique of acupuncture intervention in mice established in the previous research of this group.²⁰ In the acupuncture group, disposable sterile acupuncture needles (diameter 0.16 mm, length 7 mm, Zhongyan Taihe, China) were used to puncture ST36 on both sides of the mice, at a depth of 2–3 mm. Acupuncture was performed until a feeling

of heaviness was experienced in the hand, followed by 180° twisting manipulation, 180 times/min, twisting for 2 min, and needling once every 5 min, for a total of 4 times, and the acupuncture treatment time was 28 min. The acupuncture treatment was performed for 3 days, once a day (Figure 1).

Acupoint Injection of Drugs

HC-030031 (GLPBIO, Gc15947-50, Shanghai, China) is a TRPA1 antagonist.²¹ In this experiment, the injection concentrations were 4 mg/kg, 0.4 mg/kg, and 0.04 mg/kg, and 20 μ L was injected into ST36²² to prepare a mother liquor with a concentration of 20 mg/mL and no precipitation of the drug. Then, 2 μ L, 0.2 μ L, and 0.02 μ L of the mother liquor were dissolved in 10% DMSO+90% (20% sulfobutylether- β -cyclodextrin (SBE) in saline) (GLPBIO, GC30001-25, Shanghai, China). SBE is a derivative of sulfobutyl ether β -cyclodextrin that is commonly employed as an excipient or formulation to enhance the solubility of reagents with low solubility. Injection was performed with a micro-injector to pierce the ST36, and 10 μ L of this injection was slowly introduced in multi-directional layers, the direction of the needle tip was adjusted, and the remaining medicine was slowly injected in multiple directions. The solvent group was injected with 10% DMSO+90% (20% SBE in saline) solution, as mentioned earlier.

Collagen fibers were degraded with type I collagenase (Sigma, V900891, St. Louis, MO, USA), and 20 μ L of 2.5 mg/mL of the type I collagenase was injected into the ST36. The drug dissolution method involved the use of the drug (5 mg) dissolved in 2 mL of TESCA buffer (Solarbio, R00200, Beijing, China; pH 7.4), which is commonly used for the dissolution of collagenase.

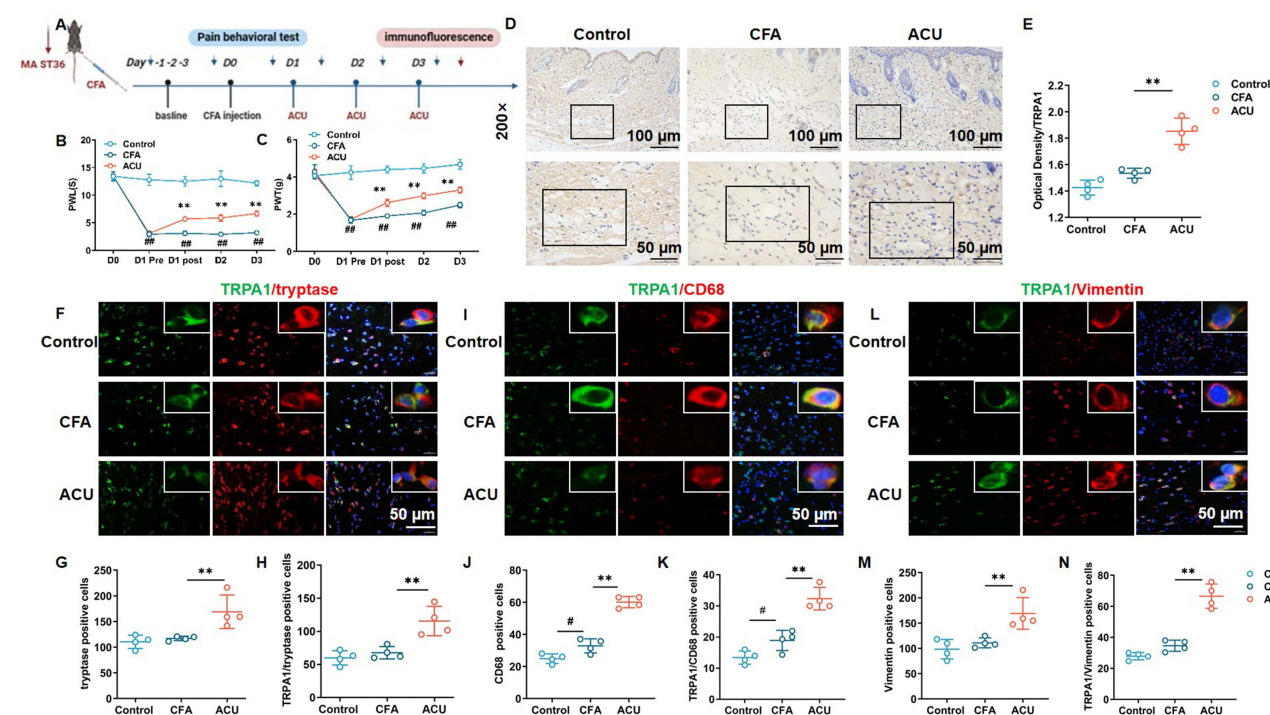


Figure 1 Acupuncture improves inflammatory pain in mice and increases the TRPA1 expression at ST36. (A) Experimental flow chart. (B) The effect of acupuncture on PWT in CFA-induced mice ($n = 8$). (C) The effect of acupuncture on PWT in CFA-induced mice ($n = 8$, CFA vs control, $^{###}p < 0.01$; ACU vs CFA, $^{**}p < 0.01$). (D) Immunohistochemical observation of the ST36 TRPA1 protein expression, scale bar = 100 μ m, 50 μ m. (E) Quantitative analysis of immunohistochemistry ($n = 4$, ACU vs CFA, $^{**}p < 0.01$). (F) Double-labeling immunofluorescence of TRPA1 (green) and mast cells (red, tryptase marker), DAPI (blue), scale bar = 50 μ m. (G) Immunofluorescence quantification of ST36 mast cells ($n = 4$). (H) Immunofluorescence quantification of ST36 TRPA1-positive mast cells ($n = 4$). (I) Double-labeling immunofluorescence of TRPA1 (green) and macrophages (red, CD68 marker), scale bar = 50 μ m. (J) Quantification of ST36 macrophages via immunofluorescence ($n = 4$). (K) Immunofluorescence quantification of ST36 TRPA1-positive macrophages cells ($n = 4$). (L) Double-labeling immunofluorescence of TRPA1 (green) and fibroblasts (red, vimentin marker), scale bar = 50 μ m. (M) Immunofluorescence quantification of ST36 fibroblasts ($n = 4$). (N) Immunofluorescence quantification of ST36 TRPA1-positive fibroblasts ($n = 4$, CFA vs control, $^{#}p < 0.05$; ACU vs CFA, $^{**}p < 0.01$).

Behavioral Testing

A BME-410C thermal radiation pain instrument (37,370, Ugo Basile, Italy) was used to measure the extent of plantar thermal radiation pain. The indoor environment was kept quiet and aglow with natural light (temperature $25 \pm 2^\circ\text{C}$, humidity $55 \pm 5\%$). Before starting the measurement, the instrument was calibrated, and a mouse was placed in a transparent grid and allowed to adapt to the environment for 30 min. After the mouse calmed down and became quiet, a radiation source was used to irradiate the heel of the mouse's foot. The interval time between the start of the irradiation and the mouse's pain avoidance response, such as paw withdrawal and PWL, was defined as the thermal pain threshold. In this study, the pain threshold was measured thrice, at an interval of 5 min, and the average value (unit: s) was taken. Thermal pain on the right plantar of mice was measured every 3 days before the modeling, 24 h after the modeling, and then 30 min after daily acupuncture. To prevent scalding of the mice, the upper limit of the thermal pain threshold was set to 30s.

When measuring the mechanical pain threshold, the probe of the electromechanical analgesia instrument pierced the sole of the mouse, and a force was applied evenly until the mouse showed a paw-withdrawal escape response. The mechanical force used at this time was defined as the PWT of the mouse. This factor reflects the mechanical pain threshold (unit: g) after the end of pain measurement, and the measurement time is consistent with thermal pain.²³

Immunohistochemistry

The mice were anesthetized through isoflurane inhalation, followed by perfusion of the heart with 0.9% saline and then with paraformaldehyde until the body of the mouse became stiff. Immediately, the skin and subcutaneous tissues of the mouse acupoint area were cut with an area of 2×2 mm and a depth of 2–3 mm; the collected tissues were rinsed repeatedly in phosphate-buffered saline (PBS). The mouse acupoint tissues were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin (P100928-500g, Aladdin, Shanghai, China), and sectioned to obtain a cross-section of the acupoint tissues. The sections were then routinely dewaxed to water, and endogenous peroxidase blocking agent (BOSTER, Wuhan, China) was added dropwise onto the tissues, followed by incubation at room temperature for 10 min; these sections were placed in EDTA 10X (AR0023, BOSTER, Wuhan, China) antigen retrieval solution. After heat repair in a microwave oven (D4, Galanz, Guangdong, China), 5% BSA blocking buffer (SW3015-100 mL, Solarbio, Beijing, China) was added dropwise and the tissues were incubated at 37°C for 30 min. The tissues were then treated with the primary antibody, as follows. TRPA1 primary antibody (1:200 dilution, NB110-40,763, NOVUS, St. Louis, MO, USA) was added dropwise at 4°C overnight, and washed with the PBS washing solution (AR0030, BOSTER, Wuhan, China) for 5 min, thrice. For the secondary antibody treatment, polymeric anti-rabbit IgG-HRP (1:100, SV0002, BOSTER, Wuhan, China) secondary antibody was dropped onto the slide and allowed to reach the room temperature for 30 min, followed by washing thrice with PBS for 5 min. The DAB (AR1022, BOSTER, Wuhan, China) chromogenic solution was added dropwise onto the slide, and the corresponding protein was observed in the slice. Mayer's (AR0005, BOSTER, Wuhan, China) hematoxylin was added dropwise onto the slide and incubated at 37°C for 1 min. The slide was then washed with PBS (pH 7.2–7.6) and sealed, as follows. The alkaline solution turned blue, and the experimental slice was sealed with neutral gum (AR0038, BOSTER, Wuhan, China) and covered with a cover glass to avoid air bubbles. The slide was then observed under a microscope (BX51, Olympus, Tokyo, Japan) under $20\times$ and $40\times$ magnification. The positive cells appeared brownish yellow.

Immunofluorescence

For primary antibody incubation, an antibody diluent (AR1016, BOSTER, Wuhan, China) was used to dilute the primary antibody, and appropriately diluted primary antibody TRPA1 antibody (1:200), mast cell tryptase (1:100, sc-59,587, Santa, Florida, USA), vimentin (1:100, sc-373,717, Santa, Florida, USA), and CD68 (1:100, sc-20,060, Santa, Florida, USA) were used according to the double-standard matching, mixed in pairs, and treated for 4°C overnight. After removal, the tissues were rewarmed at 37°C for 30 min and washed thrice with PBS for 5 min each time. Fluorescent secondary antibody incubation, DyLight 488 fluorescein (1:100, BA1127, BOSTER, Wuhan, China) labeled goat anti-rabbit and fluorescent DyLight 594 (1:100, BA1127, BOSTER, Wuhan, China) labeled goat anti-mouse IgG mixed secondary antibody were added to the tissues and incubated at 37°C for 45 min. The tissues were then washed thrice with PBS (pH 7.2–7.6) for 5 min each time. DAPI is a commonly used blue fluorescent DNA dye that can penetrate cell

membranes. DAPI staining solution (AR1176, BOSTER, Wuhan, China) was added dropwise onto the slides and incubated at room temperature for 3 min, followed by washing with PBS. Anti-fluorescence quenching mounting medium (AR1109, BOSTER, Wuhan, China) was added dropwise. Fluorescence microscope observation results and photographs or a scanner (APERIO VERSA 8, Leica, Europe) at 10×40 times were used for panoramic scanning. Under the scanner, the TRPA1 protein appears as green fluorescence, and mast cells, macrophages, and fibroblasts appear as red fluorescence. After the application of an Image J 1.8.0 software merge, the orange fluorescence portion in the picture was detected to be the co-stained cell. Each sample randomly intercepted 3 fields of view. Image J 1.8.0 software was used to count the cells of the two channels separately to obtain the region of interest (ROI) of the cells in the two channels, followed by calculation of the overlap of the ROI of the two channels and cell enumeration.

Statistical Analysis

The data were expressed as the mean \pm standard error,²⁴ and SPSS 21.0 software was used for statistical analysis. For data satisfying the normal distribution, a one-way analysis of variance was applied, and, for those that did not, the non-parametric method was applied for statistical analysis. Under the premise of satisfying the normal distribution, if the variances were homogeneous, the least significant difference (LSD) test was adopted; if the variances were not homogeneous, Dunnett's T3 test was performed. Multiple groups were analyzed using the repeated measures method. $p < 0.05$ was considered to be statistically different, and $p < 0.01$ was considered to indicate a significant statistical difference.²⁵

Results

Acupuncture Improves Inflammatory Pain in Mice and Increases TRPA1 Expression at ST36

TRPA1 participates in anti-inflammatory responses, which may be associated with the local activation of ST36 immune cells. However, the role of TRPA1 in acupuncture-led improvement of anti-inflammatory analgesia has rarely been reported. We first measured PWL and PWT to evaluate the degree of hyperalgesia induced by CFA injection and the anti-analgesic effect of acupuncture. In the CFA group vs the control group, the pain threshold of mice decreased on days 1, 2, and 3. In the ACU group vs the CFA group, the PWL and PWT of the mice increased significantly on days 1, 2, and 3 ($p < 0.01$, Figure 1A–C). In the CFA group vs the control group, the positive expression of TRPA1 at the ST36 did not change significantly ($p > 0.05$). In the ACU group vs the CFA group, the positive expression of TRPA1 was upregulated significantly ($p < 0.05$, Figure 1D–E).

We further observed the cellular localization of TRPA1 via immunofluorescence. In the CFA group vs the control group, the number of mast cells at the ST36 exhibited an upward trend ($p > 0.05$, Figure 1F and G). In the ACU group vs the CFA group, the number of mast cells at the ST36 increased significantly, while the number of TRPA1-positive mast cells increased significantly ($p < 0.01$, Figure 1H). In the CFA group vs the control group, the macrophages at the ST36 increased ($p < 0.05$, Figure 1I and J) and the number of TRPA1-positive macrophages at the ST36 showed an upward trend ($p < 0.05$, Figure 1K). In the ACU group vs the CFA group, the number of macrophages at the ST36 increased significantly ($p < 0.01$) and the number of TRPA1-positive macrophages at the ST36 increased significantly ($p < 0.01$). In the CFA group vs the control group, the number of fibroblasts at the ST36 showed an upward trend ($p > 0.05$, Figure 1L and M) and the number of TRPA1-positive fibroblasts at the ST36 tended to increase ($p > 0.05$, Figure 1N). In the ACU group vs the CFA group, the number of fibroblasts at the ST36 increased significantly ($p < 0.01$), while the number of TRPA1-positive fibroblasts at the ST36 increased significantly ($p < 0.01$).

TRPA1 Gene Deletion in Mice Reduces the Analgesic Effect of Acupuncture

Our results revealed that the TRPA1 expression at the ST36 increased after acupuncture. Therefore, we investigated whether the deletion of TRPA1 affected the analgesic effect of acupuncture. We noted that KO and WT mice exhibited no significant difference between PWL and PWT in the control group, and CFA decreased hyperalgesia in the KO and WT mice ($p < 0.05$, Figure 2A and B). In the TRPA1^{-/-} CFA vs TRPA1^{+/+} CFA groups, PWL increased on day 3 ($p < 0.05$) and PWT increased on days 2 and 3 ($p < 0.05$), indicating that KO mice experienced an analgesic effect after model.

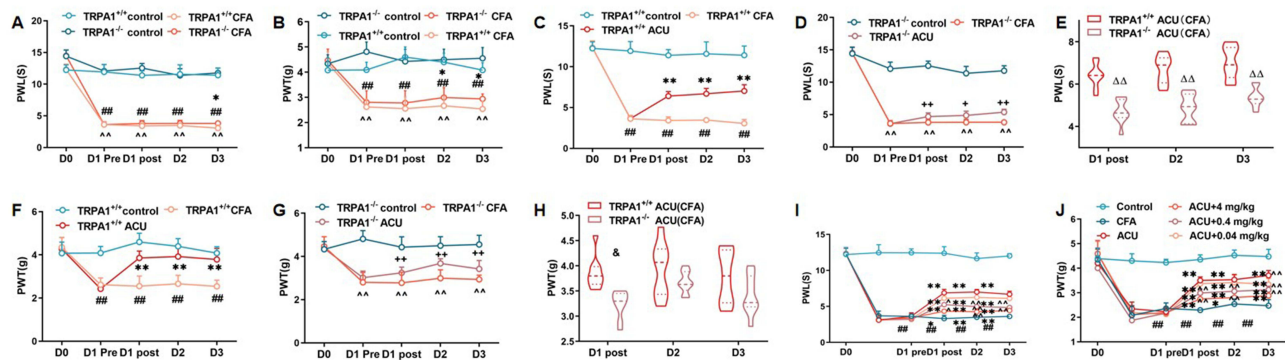


Figure 2 TRPA1 KO mice experienced reduced analgesic effect of acupuncture. (A) KO and WT mice PWL induced by CFA ($n = 8-9$). (B) KO and WT mice PWT induced by CFA ($n = 8-9$, TRPA1^{+/+} CFA vs TRPA1^{+/+} control, ### $p < 0.01$; TRPA1^{-/-} CFA vs TRPA1^{-/-} control, ^^ $p < 0.01$; TRPA1^{-/-} CFA vs TRPA1^{+/+} CFA, * $p < 0.05$). (C and D) The effect of acupuncture on PWL in CFA-induced KO and WT mice ($n = 8-9$, TRPA1^{+/+} ACU vs TRPA1^{+/+} CFA, ** $p < 0.01$; TRPA1^{-/-} ACU vs TRPA1^{-/-} CFA, * $p < 0.05$, ** $p < 0.01$). (E) The effect of acupuncture on PWL in CFA-induced KO and WT mice ($n = 8-9$, TRPA1^{-/-} ACU vs TRPA1^{+/+} ACU, $\Delta\Delta$ $p < 0.01$). (F and G) The effect of acupuncture on PWT in CFA-induced KO and WT mice ($n = 8-9$). (H) The comparison of the effect of CFA-induced acupuncture on PWT between KO and WT mice ($n = 8-9$, TRPA1^{-/-} ACU vs TRPA1^{+/+} ACU, & $p < 0.05$). (I) The effect of HC-030031 on PWT enhancement of acupuncture in CFA mice ($n = 6$). (J) The effect of HC-030031 on PWT enhancement of CFA mice by acupuncture ($n = 6$, CFA vs control, ### $p < 0.01$; ACU vs CFA, * $p < 0.05$, ** $p < 0.01$; ACU + high-, ACU + medium-, ACU + low-dose vs ACU, ^ $p < 0.05$, ^^ $p < 0.01$).

When compared with the CFA group, the pain threshold of the TRPA1^{+/+} ACU group mice increased after days 1, 2, and 3 ($p < 0.01$, Figure 2C), whereas the pain threshold of TRPA1^{-/-} ACU mice increased on days 1 and 3 ($p < 0.01$) and day 2 ($p < 0.05$, Figure 2D). In the TRPA1^{-/-} ACU group vs TRPA1^{+/+} ACU group, PWL decreased on days 1, 2, and 3 ($p < 0.01$, Figure 2E), by 27%, 27%, and 24%, respectively. The pain threshold of the mice in the TRPA1^{+/+} ACU group increased ($p < 0.01$, Figure 2F), and the pain threshold of those in the TRPA1^{-/-} ACU group PWT increased on days 1, 2, and 3 ($p < 0.01$, Figure 2G). In the TRPA1^{-/-} ACU group vs TRPA1^{+/+} ACU group, PWT decreased on day 1, 2 and 3 ($p < 0.05$, Figure 2H), by 16%, 6%, and 10%, respectively.

To determine whether the local TRPA1 in the ST36 is involved in the initiation of the acupuncture effect, we injected the HC-030031 antagonist locally at the ST36. In the CFA group vs the control group, we noted that the PWL decreased significantly on days 1, 2, and 3 ($p < 0.01$, Figure 2I). In the ACU + high-, ACU + medium-, and ACU + low-dose groups vs the CFA group, the PWT increased significantly on days 2 and 3 ($p < 0.01$, Figure 2I) and increased on day 1 ($p < 0.05$). In the ACU + high- and ACU + medium-dose groups vs ACU group, the PWL decreased significantly on days 1, 2, and 3 ($p < 0.01$, Figure 2I). The ACU + low-dose group vs the ACU group showed a downward trend on days 1 and 3 ($p > 0.05$, Figure 2I). In the CFA group vs the control group, the PWT decreased significantly on days 1, 2, and 3 ($p < 0.01$, Figure 2J). In the ACU + high-, ACU + medium-, and ACU + low-dose groups vs the CFA group, the PWT increased significantly on days 1 and 3 ($p < 0.01$, Figure 2J) and increased on day 2 ($p < 0.05$). In the ACU + high- and ACU + medium- dose groups vs the ACU group, the PWT decreased significantly on days 1, 2, and 3 ($p < 0.01$, Figure 2J). The ACU + low-dose group vs the ACU group showed a downward trend on days 1 and 2 ($p > 0.05$).

Similarly, in the carrageenan-induced inflammatory pain model, carrageenan (Car) decreased hyperalgesia in the KO and WT mice, TRPA1 KO mice experienced reduced analgesic effect of acupuncture. In the TRPA1^{-/-} Car group vs TRPA1^{+/+} Car group, PWL increased significantly on days 1 and 3 ($p < 0.05$, Figure 3A). PWT showed an upward trend, and a significant statistical difference was noted on days 2 and 3 ($p < 0.01$, Figure 3B). After acupuncture treatment, the PWL of the KO and WT mice showed a gradual increase compared with that of the Car group ($p < 0.01$, Figure 3C and D). In the TRPA1^{-/-} ACU group vs the TRPA1^{+/+} ACU group, the PWL decreased on days 1 and 3 ($p < 0.01$, Figure 3E) and day 2 ($p < 0.05$). The PWL decreased by 17%, 12%, and 20%, respectively. After acupuncture, the PWT of the KO and WT mice gradually increased when compared with that of those in the model group, and the pain threshold of the TRPA1^{+/+} ACU mice increased ($p < 0.01$, Figure 3F), whereas the pain threshold of the TRPA1^{-/-} ACU mice was significantly improved on days 1 ($p < 0.01$) and 3 ($p < 0.05$, Figure 3G). In the TRPA1^{-/-} ACU group vs the TRPA1^{+/+} ACU group, the PWT decreased on days 1 and 2 ($p < 0.01$, Figure 3H); the PWT decreased by 15%, 13%, and 6%, respectively.

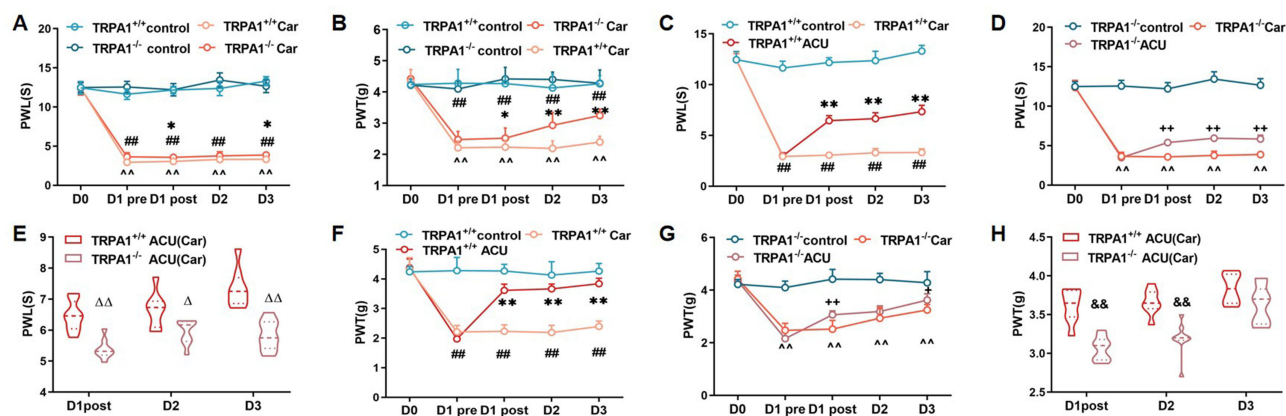


Figure 3 TRPA1 KO mice experienced reduced analgesic effect of acupuncture. (A) Carrageenan-induced PWL between KO and WT mice ($n = 8-9$). (B) Carrageenan-induced PWT between KO and WT mice ($n = 8-9$, TRPA1^{+/+} Car vs TRPA1^{+/+} control, ### $p < 0.01$; TRPA1^{-/-} Car vs TRPA1^{-/-} control, ^^ $p < 0.01$; TRPA1^{-/-} Car vs TRPA1^{+/+} Car, * $p < 0.05$, ** $p < 0.01$). (C and D) The effect of acupuncture on PWL in Car-induced KO and WT mice ($n = 8-9$, TRPA1^{+/+} ACU vs TRPA1^{+/+} Car, ** $p < 0.01$; TRPA1^{-/-} ACU vs TRPA1^{-/-} Car, ++ $p < 0.01$). (E) The comparison of the effect of Car-induced acupuncture on PWL between KO and WT mice ($n = 8-9$, TRPA1^{-/-} ACU vs TRPA1^{+/+} ACU, Δ $p < 0.05$, ΔΔ $p < 0.01$). (F and G) The effect of acupuncture on PWT in Car-induced KO and WT mice ($n = 8-9$). (H) Comparison of the effect of Car-induced acupuncture on PWT between KO and WT mice ($n = 8-9$, TRPA1^{-/-} ACU vs TRPA1^{+/+} ACU, && $p < 0.01$).

Injecting Collagenase at the ST36 Partly Antagonizes the Analgesic Effect of Acupuncture and Downregulates the TRPA1 Expression

Previous studies have reported that collagenase can be used to destroy collagen fibers at the ST36 of rats, and acupuncture could not effectively induce mast cell degranulation, the analgesic effect was significantly attenuated.³ Therefore, we determined whether collagen fibers are the trigger condition affecting TRPA1 activation. We found that CFA decreased the PWL and PWT of mice, and the PWL and PWT of the model side increased significantly after acupuncture ($p < 0.01$, Figure 4A). In the ACU + collagenase group vs the ACU + TESCA group, PWL decreased significantly ($p < 0.01$), and PWT decreased significantly on day 1 and day 3 ($p < 0.01$), and day 2 ($p < 0.05$, Figure 4B). These findings indicate that collagenase partly antagonizes the analgesic effect of acupuncture.

We co-stained the TRPA1 protein and the expressed cells by immunofluorescence. In the ACU + collagenase group vs ACU + TESCA, the positive expression of TRPA1 was downregulated ($p < 0.01$, Figure 4C and D). The number of mast cells decreased significantly ($p < 0.05$, Figure 4E), and the number of co-stained TRPA1 and mast cells was downregulated ($p < 0.01$, Figure 4F). The number of macrophages was significantly downregulated ($p < 0.01$, Figure 4G and H), and the number of co-stained TRPA1 and macrophages in the ST36 was significantly downregulated ($p < 0.01$, Figure 4I). The number of fibroblasts was significantly downregulated ($p < 0.01$, Figure 4J and K), and the number of co-stained TRPA1 and fibroblasts in the ST36 was significantly downregulated ($p < 0.01$, Figure 4L).

Discussion

Acupuncture exhibits distinct anti-inflammatory and analgesia effects.^{26,27} The inflammatory response plays a crucial role in activating acupuncture points. Various mechanisms interact to form a small network of acupuncture points. The local acupoint is an inflammatory reaction area. Acupuncture stimulation, a mildly damaging stimulus, can induce a small-scale inflammatory reaction at the local inflammation site.^{28,29} Damage, congestion, and edema can form in the tissue of the acupoint region. Recruitment of immune cells and activation of inflammation-related signaling pathways produce inflammatory mediators, such as tumor necrosis factor- α (TNF- α) and interleukin- β (IL- β), leading to the occurrence of inflammatory reactions to activate the local immune response. However, this immune response cannot amplify indefinitely nor can it trigger a systemic inflammatory response. At the later stage of acupuncture, this inflammatory response gradually subsides, and anti-inflammatory factors can be produced to protect against tissue damage.^{30,31} This localized inflammatory response can be one of the key factors for the activation of acupoints. Recent studies have indicated that acupuncture can cause the deformation of collagen fibers and activate TRP channels on cells membrane and releases various biological substances such as ATP, adenosine, damage-associated molecular patterns (DAMPs), and other substances, the analgesic effect of acupuncture is

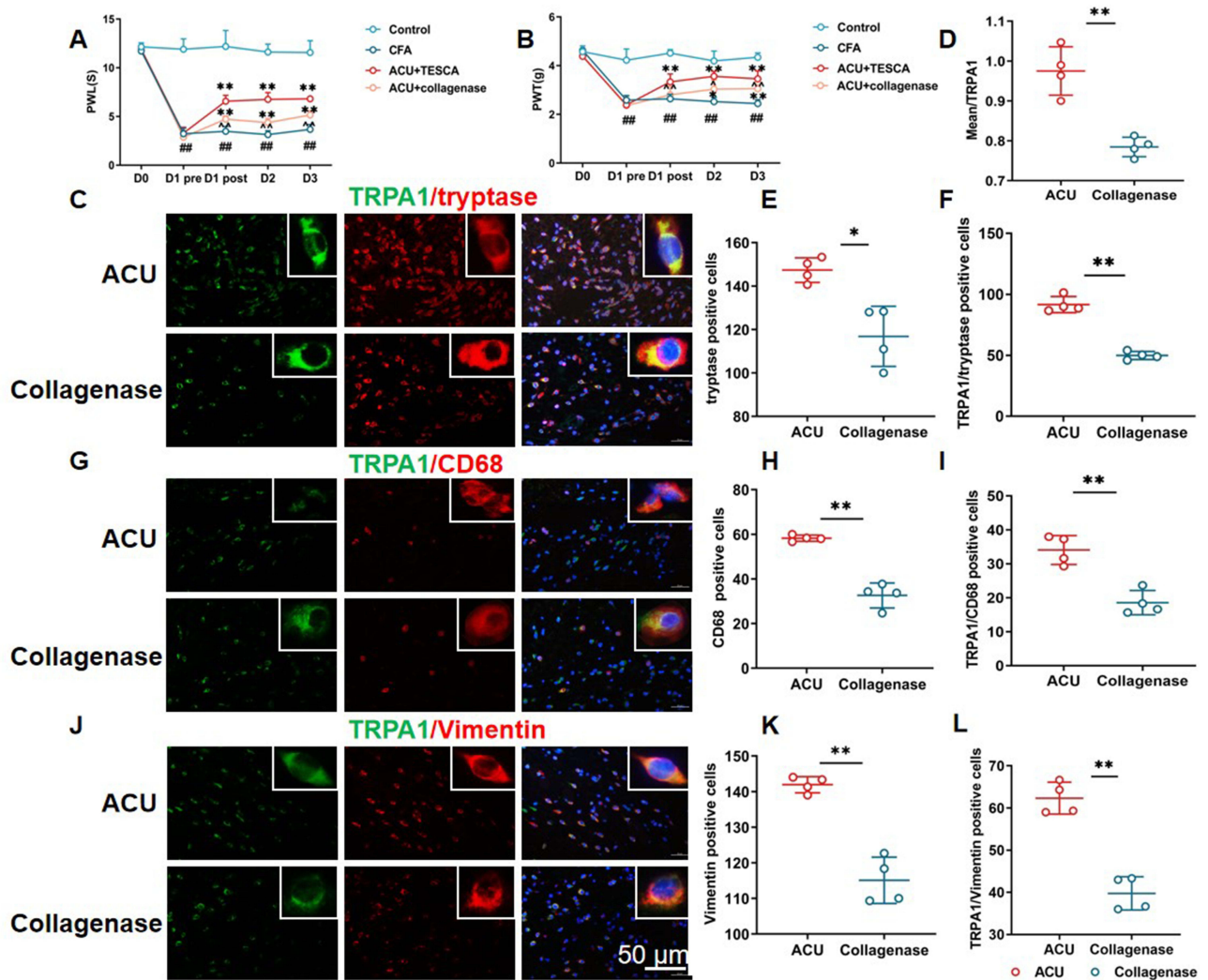


Figure 4 ST36 injection of collagenase partly antagonizes the analgesic effect of acupuncture, and the expression of TRPA1 protein decreases. (A) The effect of collagenase injection on PWL enhancement of CFA mice by acupuncture ($n = 6$). (B) The effect of collagenase injection on PWT enhancement of CFA mice by acupuncture ($n = 6$, CFA vs control, $###p < 0.01$; ACU vs CFA, $*p < 0.05$, $**p < 0.01$; ACU + collagenase vs ACU, $^{\wedge}p < 0.05$, $^{\Delta}p < 0.01$). (C) Double-labeling immunofluorescence of TRPA1 (green) and mast cells (red, tryptase marker), DAPI (blue), scale bar = 50 μm . (D) The quantification of the ST36 TRPA1 protein expression by immunofluorescence ($n = 4$). (E) Immunofluorescence quantification of ST36 mast cells ($n = 4$). (F) Immunofluorescence quantitative analysis of ST36 TRPA1-positive mast cells ($n = 4$). (G) Double-labeling immunofluorescence of TRPA1 (green) and macrophages (red, CD68 marker), scale bar = 50 μm . (H) The quantification of ST36 macrophages via immunofluorescence ($n = 4$). (I) Immunofluorescence quantitative analysis of ST36 TRPA1-positive macrophages ($n = 4$). (J) Double-labeling immunofluorescence of TRPA1 (green) and fibroblasts (red, vimentin marker), scale bar = 50 μm . (K) Immunofluorescence quantitative analysis of ST36 fibroblasts ($n = 4$). (L) Immunofluorescence quantitative analysis of ST36 TRPA1-positive fibroblasts ($n = 4$, ACU vs ACU + collagenase, $*p < 0.05$, $**p < 0.01$).

related to the structure of collagen fibers.³² Macrophages, fibroblast membrane surface receptors, and the local inflammatory response at the acupoint area participate in the transduction of acupuncture signals and play a key role as the source link at acupuncture. Mast cells serve as sentinels of innate immunity and regulators of adaptive immunity.^{33,34} The function of mast cells is mainly realized through the process of degranulation; after degranulation, abundant mediators are released, including histamine, platelet-activating factor (PAF), prostaglandin D2 (PGD2), SP, tryptase, serotonin, and chemokines.³⁵ Owing to the distribution of mast cells, these mediators can quickly act on the adjacent nerves, blood vessels, and muscles to form a neuro-endocrine-immune network. After acupuncture stimulation, mast cells can migrate, aggregate, and activate.³⁶ Previous studies have demonstrated that mast cells can be activated via physical stimulation in a *Xenopus* oocyte model on the activation of TRPV1 by 1 μM capsaicin-induced mast cell degranulation.³⁷ The activation of TRPV1, TRPV2, and TRPV4 contributes to the activation of mast cells induced via acupuncture stimulation. The associated degranulation contributes to the initial step of analgesia.³⁸ Fibroblasts are the most common cells in connective tissues and the key cells involved in local immune

responses.³⁹ Tissue damage stimulates fibroblasts and induces fibroblast mitosis. Tissue damage produces several substances that stimulate the activation of fibroblasts and release inflammatory factors; therefore, regulating fibroblasts can control the occurrence and resolution of inflammation. Chen et al reported that fascial fibroblasts at the ST36 can directly lead to the mechanical stimulation of acupuncture, converting it into a biological signal while increasing the content of prostaglandin E2 (PGE2).⁸ Acupuncture stimulation increases the number of fibroblasts and the amount of adenosine in fibroblasts via energy metabolism pathways.⁴⁰ Macrophages, which play a key role in maintaining tissue homeostasis, play a crucial role in the pathophysiological processes of tissue damage, repair, and inflammatory responses. Past studies have shown that local macrophages at acupoints increase after acupuncture.⁴¹ Xu et al employed high-throughput technology and found that macrophages are the key cells that respond to acupuncture effects.⁴²

The TRPA1 channel can be activated by cold stimulation, mechanical stimulation, and various inflammatory mediators. Its function is regulated by numerous factors, including Ca^{2+} , pH, ROS, and nitrogen.^{43,44} As a pain sensor highly expressed in C fibers linked to neuropathic pain, TRPA1 is primarily associated with neurogenic inflammation,⁴⁵ and it exhibits biological effects mediating both inflammation and pain. TRPA1 channels are regulated in two ways in the context of inflammatory pain. On one hand, the activation of TRPA1 channels promotes the release of inflammatory factors, thereby increasing the nociceptor sensitivity. On the other hand, TRPA1 stimulation is often correlated with the release of SP or CGRP. These neuropeptides coordinate a feed-forward pro-inflammatory mechanism via the promotion of vasodilation, plasma protein leakage, and immune cell stimulation. TRPA1 activation and sensitization mediates skin inflammation by increasing the release of inflammatory mediators; conversely, inflammatory factors mediate the sensitization of TRPA1 within cells, thereby promoting its activation.⁴⁶ TRPA1 has been reported to be predominantly expressed in nerve fibers and immune cells, thus serving as the first line of defense against tissue damage and pathogen invasion. In cases of tissue injury, TRPA1 is activated to trigger an immune response. In various inflammatory diseases, TRPA1 plays an anti-inflammatory role by inhibiting nuclear factor kappa B (NF- κ B), reducing the release of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX2), TNF- α expression in macrophages, and subsequent nitric oxide (NO) production, and regulating T-cell activation. TRPA1-KO mice exhibit increased macrophage infiltration and renal dysfunction.⁴⁷ TRPA1 also protects the kidney from damage associated with sepsis.⁴⁸ The upregulation of TRPA1 in an atherosclerotic plaque model mediated the transition of macrophages to an anti-inflammatory phenotype, leading to the attenuation of atherosclerosis.⁴⁹ Furthermore, TRPA1 activation can improve insulin signaling and mitochondrial function, reduce obesity-related inflammation, and lower the levels of TNF- α .⁵⁰ The deletion of TRPA1 in mouse CD4⁺ T cells induced the upregulation of pro-inflammatory factors, including TNF- α .⁵¹ Capsazepine activates TRPA1, followed by significant sustained desensitization and subsequent anti-inflammatory effects.⁵² In addition, the study found that TRPA1 interacts with members of the interleukin (IL) family during inflammation.⁵³ TRPA1 mediates IL-6 expression in human and mouse chondrocytes. Cannabinoids exert anti-inflammatory effects in rheumatoid arthritis by activating TRPA1 and reducing IL-6 production in synovial fibroblasts.⁵⁴ Endogenous opioid peptides such as beta-endorphins are released by neutrophils and mononuclear macrophages and bind to peripheral μ id receptors (MOR). Naloxone, an antagonist of MOR, has been found to block TRPA1 blockade by HC-030031 in vivo inflammation and TRPA1-mediated calcium influx in DRG in vitro experiments.⁵⁵ Enterochromaffin (EC) cells release serotonin (5-serotonin; 5-HT) stimulates nerves and is associated with gastrointestinal function, and AITC, an agonist of TRPA1, can increase intracellular Ca^{2+} levels and 5-HT release.⁵⁶ The study found that TRPA1 and 5-HT induce nociception in an interdependent manner, can increase the sensitivity of nociceptors to inflammatory mediators.⁵⁷

We initially employed immunohistochemistry to confirm that TRPA1 was predominantly distributed in the dermis and connective tissue layers of the acupoints, with lower expression observed in the muscle layer. Building upon this observation, we further elucidated the cell types expressing TRPA1. We conducted immunofluorescence co-localization studies using markers of key cells in the acupoint area and found that TRPA1 co-localized with markers for mast cells, macrophages, and fibroblasts. These cells primarily exhibited expression in the cell membrane and cytoplasm within the dermis and connective tissue layer of the acupoint area. After acupuncture treatment, the mast cells tended to aggregate nearer to the acupuncture site, accompanied by degranulation, and their numbers significantly increased. Macrophages also significantly increased and were primarily distributed in the dermis of the acupoint skin. Fibroblasts exhibited a more closely arranged, spindle-shaped cytoskeleton, leading to an increased cross-sectional area.

Using transgenic animals, we investigated whether TRPA1 is involved in the analgesic effects of acupuncture. We initially assessed the baseline pain threshold of KO mice and WT mice under physiological conditions, and, while the pain threshold of KO mice was slightly higher than that of WT mice, this difference was not statistically significant. The pain threshold gradually stabilized on days 2 and 3, which may be attributed to environmental and mouse-specific factors during measurement. Following the induction of inflammation in KO mice using CFA, we observed an upward trend in heat pain compared to that in the WT model group. KO mice displayed relief from persistent inflammation-related mechanical sensitivity, which is consistent with the findings of Moilanen et al^{58–60} However, this change was not evident

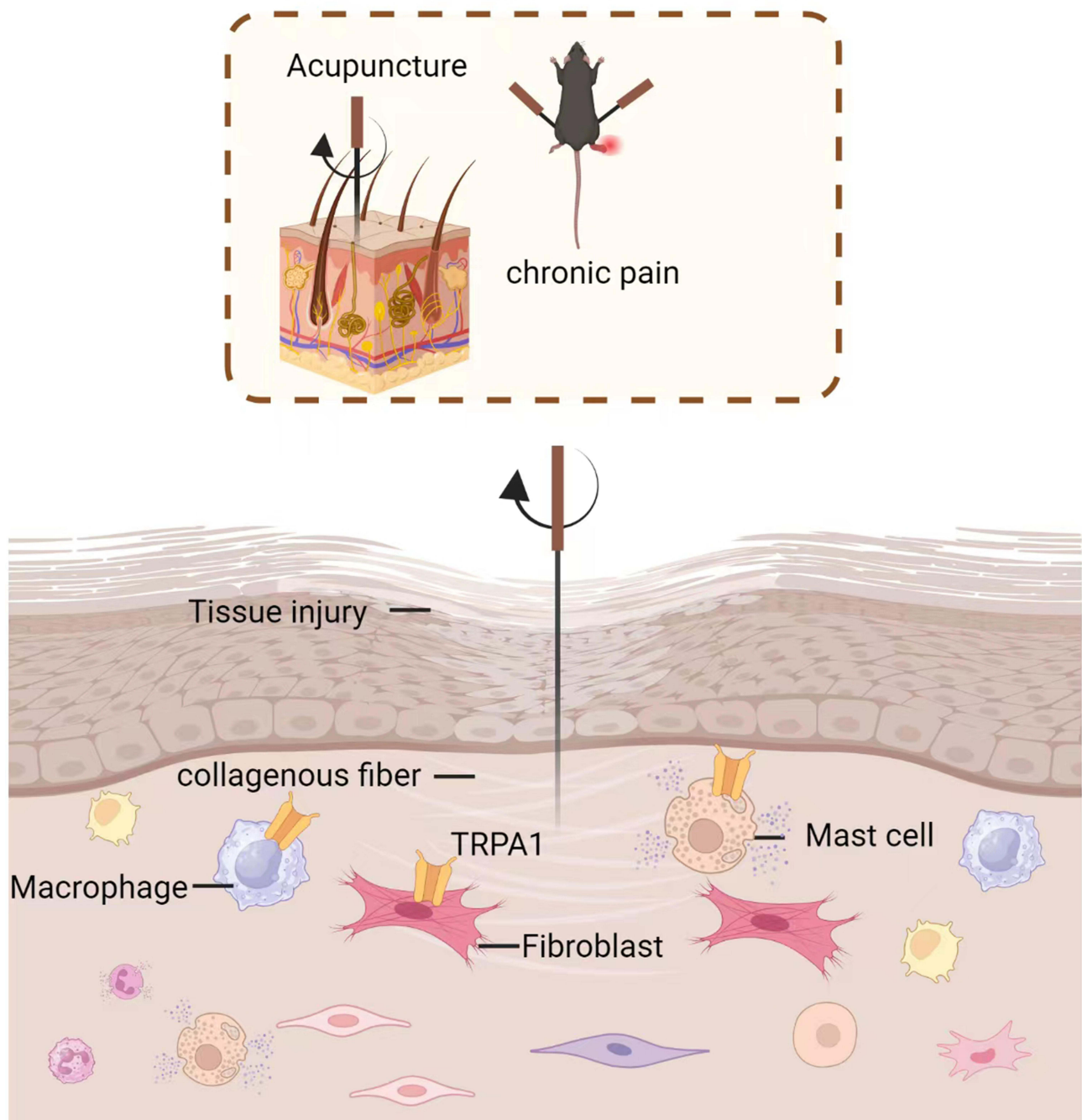


Figure 5 The involvement of TRPA1 in the initiation mechanism of acupuncture ST36 for alleviating arthritis pain in mice. Mechanical stimulation by acupuncture causes winding of collagen fibers in the acupoint area, and stimulates TRPA1 ion channels in mast cells, macrophages, and fibroblast cell membranes, converting mechanical signals into bioelectrical signals. (The arrows represent a larger view of ST36.).

on the first day of modeling, aligning with previous reports.⁶¹ Upon acupuncture intervention in both the models, we noted an increase in the pain threshold at the affected side and an improvement in inflammatory pain in both sets of mice. Comparing the acupuncture effects between the two groups, we found that under both inflammatory pain models, the analgesic effect of acupuncture in KO mice decreased, which indicated that TRPA1 is involved in the analgesic effects of acupuncture. We subsequently validated these results through pharmacological experiments by locally injecting channel antagonist HC-030031 with varying concentrations of TRPA1 in the acupoint area and found that the analgesic effect of acupuncture was partially antagonized. Therefore, we hypothesized that TRPA1 plays distinct roles in different contexts, participating in localized pain and localized analgesia at acupuncture points. Although there have been studies injecting TRPA1 antagonists into acupuncture points and paws, which have initially shown that TRPA1 is activated after receiving acupuncture stimulation and exerts an analgesic effect locally in the acupoint areas, there are few relevant literature reports, and the types of cells in the acupoint areas where it is expressed and triggering factors are unclear and have not been verified using genetically modified animals.⁶² This study identified the cell types expressing TRPA1 in ST36 and utilized three concentrations of antagonists and KO mice to confirm the involvement of TRPA1 in acupuncture analgesia. Additionally, the study investigated the upstream substances regulating TRPA1.

Consequently, mechanical stimulus by acupuncture forms a small network within acupoints and dynamically regulates local cell communication networks in inflamed joints, ultimately resulting in an analgesic effect (Figure 5). Our findings indicate the crucial role of TRPA1 in the activation of signaling pathways associated with tissue damage. However, the existing literature indicates that the role of TRPA1 in the inflammatory response is two-fold. On one hand, it serves a defensive role against cellular stress, tissue damage, and harmful stimuli. On the other hand, TRPA1 may exacerbate inflammation, which may depend on the specific states of inflammatory diseases. The precise mechanism underlying the anti-inflammatory role of TRPA1 in intracellular signaling pathways remains elusive. A comprehensive understanding of TRPA1 in terms of its specific action pathways, associated upstream and downstream mechanisms, and regulation within the peripheral and central nervous and immune systems is lacking. Modulating ion-channel activity represents a dynamic and intricate process influenced by various factors, including inflammatory mediators, intervention methods, stimulus intensity, and the status of the nervous system.

Conclusions

This study explored the role of TRPA1 and key immune cells in the activation of ST36. We employed techniques such as gene knockout and a specific channel antagonist to investigate the analgesic effects of acupuncture triggered by changes in acupoint TRPA1 expression as well as studying the upstream conditions that activate TRPA1. Our findings indicate that TRPA1 plays a crucial role in the localized analgesic effects induced by acupuncture at acupoints.

Abbreviations

ATP, Adenosine triphosphate; CPZ, Capsazepine; CGRP, Calcitonin gene-related peptide; CFA, Complete Freund adjuvant; COX2, Cyclooxygenase-2; DAMPs, Damage-associated molecular patterns; EC, Enterochromaffin; 5-HT, 5-serotonin; IL-1 β , Interleukin-1 β ; iNOS, Inducible nitric oxide synthase; LSD, Least significant difference; MSc, Mechanosensitive channel; NF- κ B, Nuclear factor kappa B; NO, Nitric oxide; PAF, Platelet-activating factor; PWL, Paw withdrawal latency; PWT, Paw withdrawal threshold; PNS, Peripheral nervous system; PKC, Protein kinase C; ROS, Reactive oxygen species; RA, Rheumatoid arthritis; SP, Substance P; SPF, Specific-pathogen-free; ST36, Zusanli; TRPV1, Transient receptor potential vanilloid 1; TRPA1, Transient receptor potential ankyrin 1; TNF- α , Tumor necrosis factor- α ; WT, Wild-type.

Institutional Review Board Statement

The animal study protocol was approved by the Tianjin University of Traditional Chinese Medicine of NAME OF INSTITUTE (protocol code TCM-LAEC20211279 and date of approval) for studies involving animals.

Data Sharing Statement

All data are in this article.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors state that this study was conducted without any commercial or financial relationships which could be interpreted as potential conflicts of interest.

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