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Transient receptor potential ankyrin 1 in the knee is involved in osteoarthritis pain

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ABSTRACT

Transient receptor potential families play important roles in the pathology of osteoarthritis (OA) of the knee. While transient receptor potential ankyrin 1 (TRPA1) is also an essential component of the pathogenesis of various arthritic conditions, its association with pain is controversial. Thus, we researched whether TRPA1 is involved in knee OA pain by *in vivo* patch–clamp recordings and evaluated the behavioral responses using CatWalk gait analysis and pressure application measurement (PAM). Injection of the *Trpa1* agonist, allyl isothiocyanate (AITC), into the knee joint significantly increased spontaneous excitatory synaptic current (sEPSC) frequency in the substantia gelatinosa of rats with knee OA, while injection of the *Trpa1* antagonist, HC-030031, significantly decreased the sEPSC. Meanwhile, AITC did not affect the sEPSC in sham rats. In the CatWalk and PAM behavioral tests, AITC significantly decreased pain thresholds, but no difference between HC-030031 and saline injections was observed. Our results indicate that *Trpa1* mediates knee OA-induced pain. We demonstrated that *Trpa1* is activated in the knee joints of rats with OA, and *Trpa1* activity enhanced the pain caused by knee OA.

1. Introduction

Impaired mobility in older adults is predominantly attributed to osteoarthritis (OA), one of the most common conditions treated in clinics worldwide [1]. Patients with OA mainly complain about pain, particularly when walking and stair climbing [2]. OA is a growing socioeconomic concern and is increasing in prevalence worldwide due to population aging [3]. The knee OA stage is determined according to the knee OA severity classification, but conflicting reports between clinical symptoms and radiographs have been described [4,5]. However, it is widely known that most individuals with radiography-confirmed knee OA are asymptomatic [6]. The underlying mechanisms of pain in knee OA are unclear, and asymptomatic radiographic knee OA remains controversial.

The transient receptor potential (TRP) ion channel family comprises homotetrameric and heterotetrameric polypeptides that are expressed throughout the body and play a crucial role in regulating homeostasis [7,8]. TRP ankyrin 1 (TRPA1) is an ion channel that is permeable to many cations discovered in 1999, expressed mainly in the sensory nervous system, and together with TRP vanilloid 1 mediates nociception, hyperalgesia, and neurogenic inflammation as a nociceptive chemosensor [9–13]. While it is broadly expressed in the neurons, TRPA1 has also been found to be expressed in non-neuronal cells such as those in the lung, inner ear, and intestinal tract, as well as skin keratinocytes, vascular endothelial cells, and synoviocytes [9,14,15]. As such, TRPA1 is a promising target in the treatment of pain and inflammation-related conditions, such as inflammatory arthritis [16–18]. In knee OA models, TRPA1-mediated pain was associated with inflammation and cartilage degradation [19,20]; however, further research is required to determine the mechanisms underlying these favorable effects.

In this study, we focused on the functional effects of *Trpa1* in a rat model of knee OA. Knee joint nociceptive signals were analyzed by recording spontaneous excitatory synaptic currents (sEPSC) in the substantia gelatinosa (SG) using *in vivo* patch–clamp analysis. Behavioral

Abbreviations: OA, osteoarthritis; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; AITC, allyl isothiocyanate; sEPSC, spontaneous excitatory synaptic currents; PAM, pressure application measurement; SG, substantia gelatinosa; OARSI, Osteoarthritis Research Society International.

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experiments were performed on the OA rats using the *Trpa1* agonist, allyl isothiocyanate (AITC), and the *Trpa1* antagonist, HC-030031, both of which are potential drug treatments for knee OA. Pressure application measurement (PAM) and CatWalk analysis of the knee joint were applied in this study to mimic clinical OA knee pain in humans.

2. Materials and methods

All the experimental protocols, including animal care, were approved by the Ethics Committee on Animal Experiments of Wakayama Medical University prior to animal use.

2.1. Knee OA model

Male Sprague–Dawley rats were used at 3 weeks of age in the patch–clamp experiments and at 5 weeks of age in the PAM and CatWalk behavioral experiments. The methods of Knee OA models in detail were used as described previously [21]. In a sham operation, a longitudinal incision was made, the patella was dislocated laterally, positioned in place, and the skin was sutured.

2.2. Histopathology scoring

Rats were sacrificed at 6 weeks following blood withdrawal by cardiac puncture. The knee joints were isolated, fixed overnight in 4% formaldehyde, and decalcified in 10% ethylenediaminetetraacetic acid (pH 7.4) for about 4–6 weeks until the bone tissue softened. The knee joint was encapsulated in paraffin, and coronal, 6-μm-thick paraffin sections were prepared. For histologic evaluation of cartilage, sections were stained with hematoxylin-eosin and safranin O fast green and scored using the Osteoarthritis Research Society International (OARSI) histopathology grading system [22,23]. At least two blinded individuals independently scored stained sections of each joint and derived an agreeable score.

2.3. In vivo patch–clamp recordings

Rats were assessed approximately 3 weeks post-surgery when degeneration of the knee had fully developed. *In vivo* patch–clamp recording methods were used as described previously [11,24,25]. After a small opening was made in the dura mater, the recording electrode was placed in the SG from the surface of the L3 or L4 spinal cord, which is considered to be innervated by the knee joint. After forming the seal, the membrane patch was ruptured by a brief period of negative pressure, resulting in a whole-cell configuration for sEPSC recordings. An indwelling needle was placed in the right knee, and the drug was administered. The activation was measured 60 s after the intra-articular administration of the drug. Once the experiments were completed, the rats were sacrificed by decapitation following a lethal dose of urethane.

2.4. CatWalk

CatWalk is a gait analysis method that automatically and objectively quantifies the parameters of each limb and those involved in coordination between limbs [26,27]. All gait parameters were analyzed with a software program dedicated to this system. Data are expressed as the ratio of the ipsilateral (right) hindlimb to the contralateral (left) hindlimb. Measurements were taken before drug administration, after 30 min, and at 1, 3, 6, 12, and 24 h.

2.5. PAM

A PAM device (Ugo Basile SRL, Italy) was used to measure mechanical pain thresholds in rats. Methods for this apparatus, including animal handling, were in accordance with those described in the aforementioned study [28]. The pain threshold was expressed as the ratio of peak gram force through the ipsilateral and contralateral limbs. Three measurements of both the ipsilateral and contralateral limbs were taken at 1 min intervals. Measurements were made before drug administration, after 30 min, and at 1, 3, 6, 12, and 24 h.

2.6. Drug application

The selective *Trpa1* agonist, AITC, and the selective *Trpa1* antagonist, HC-030031, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Given that saline and distilled water do not affect synaptic transmission or plasticity, AITC was dissolved 1000-fold in distilled water and diluted to the final concentrations in 0.9% saline for use, and HC-030031 was dissolved in 0.9% saline.

2.7. Statistical analysis

Data are expressed as the mean ± SEM. Using Mini Analysis Program 6.0 (Synaptosoft, Decatur, GA), a temporal profile of sEPSC in 30 s time bins was constructed, and the average response of 30 s at peak was used to analyze changes in the frequency and amplitude of sEPSC following AITC application. Differences were compared using Student's *t*-test. Analysis of variance (ANOVA) with repeated measures over time was used in the behavioral test and combined with a Bonferroni *post hoc* correction to detect time-related differences. All values were considered statistically significant if $p < 0.05$.

3. Results

3.1. Histological analysis of post-surgical joint changes

The median OARSI score increased significantly in the knee OA group (n = 4) compared to that in the sham group (n = 4) (Fig. 1A). Similar results were obtained for grading (Fig. 1B) and staging (Fig. 1C).

At 4 weeks post-surgery, the tibial plateaus of OA and sham animals were stained using safranin O fast green (Fig. 1D and E). The sham animals had almost normal articular surfaces and underlying subchondral bone, and the cartilage matrix was homogeneously stained (Fig. 1F and G). Compared to sham animals, the OA model showed vertical fissures in the mid-zone, where the superficial cartilage is reduced, and the distribution of the matrix is irregular. The retention of superficial features, including rougher, more fibrotic cartilage surfaces, and fewer chondrocytes in the superficial zone, was observed. This confirmed that the OA was successfully established in our models at 4 weeks post-surgery.

3.2. The effect of Trpa1 activation on sEPSC

To investigate whether *Trpa1* activity is involved in OA knee pain, we recorded sEPSC in the SG before and after injection of AITC (100 μM, 50 μL) into the OA knee joint (Fig. 2A). Before AITC administration, the average frequency of sEPSC was 4.6 ± 0.9 Hz (range: 2.1–10.5 Hz), and the amplitude of sEPSC was 10.8 ± 0.9 pA (range: 7.7–15.8 pA) (n = 7). AITC increased the proportion of sEPSC with shorter inter-event intervals but showed few changes in amplitude when compared with the control (Fig. 2C). After 30 s AITC administration, a significant increase in sEPSC frequency was noted with an average sEPSC frequency of 7.1 ± 1.1 Hz (range: 4.5–14.3 Hz) and amplitude of 11.0 ± 0.8 pA (range: 7.9–14.2 pA) (Fig. 2E). These results indicate that *Trpa1* is activated in knee OA.

When testing whether AITC activates *Trpa1* in sham-treated knee joints (Fig. 2B), AITC hardly affected on the cumulative distribution of the inter-event and amplitude of the sEPSC (Fig. 2D). Before AITC administration, an average frequency of sEPSC was 2.3 ± 0.6 Hz (range: 0.9–5.0 Hz), and the amplitude of sEPSC was 7.6 ± 0.8 pA (range: 4.7–11.6 pA) (n = 7). After 30 s AITC administration, the sEPSC had an average frequency of 2.3 ± 0.6 Hz (range: 0.9–4.5 Hz) and amplitude of 7.9 ± 0.4 pA (range: 6.8–9.9 pA); thus, no significant difference was

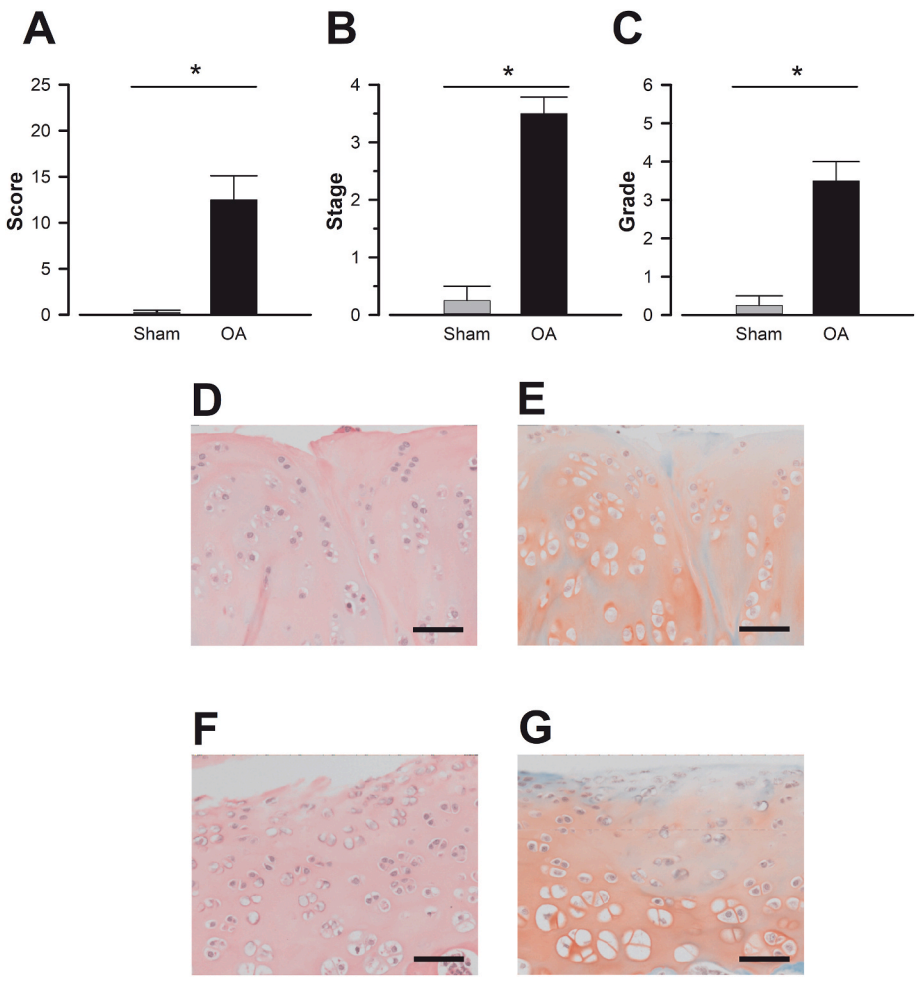

Fig. 1. Osteoarthritis Research Society International (OARSI)-modified Mankin scores of joints from rats with knee osteoarthritis (OA), and cartilage surface changes after surgery in the tibial compartment of the rat knee joint (A) OARSI score; (B) OARSI stage; (C) OARSI grade. (D–G) The tibial compartment of the rat knee joint at 4 weeks after surgery for sham animals (D, E) and the OA model (F, G), stained with hematoxylin and eosin (D, F) or safranin O fast green (E, G). Scale bar = 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

found (Fig. 2F).

The sEPSC of SG neurons of the OA rats were significantly enhanced compared with that of sham rats (Fig. 3A). To examine whether HC-030031 affects the sEPSC of SG neurons in OA rats, we injected HC-030031 (100 μM, 50 μL) into the OA knee joint (Fig. 3B). Before HC-030031 administration, the average frequency of sEPSC was 6.1 ± 1.4 Hz (range: 1.1–10.4 Hz), and the amplitude of sEPSC was 7.2 ± 0.9 pA (range: 5.2–8.3 pA) (n = 9). After 30 s HC-030031 administration, a significant decrease in the sEPSC frequency was noted with an average frequency of 3.8 ± 1.0 Hz (range: 0.8–7.6 Hz) and amplitude of 7.3 ± 0.6 pA (range: 5.4–8.2 pA) (Fig. 3D). Compared with the control, HC-030031 shifted and lengthened the inter-event interval but had few effects on amplitude (Fig. 3C). These results show that *Trpa1* is activated in knee OA and that the effect of *Trpa1* agonist was induced by *Trpa1* activation rather than by the side effect.

3.3. Comparison of paw print area and standing time using the CatWalk system

The CatWalk test is suitable for assessing chronic pain, such as in OA [26]. In this study, it was used to record standing time (stand) and maximum paw area (max contact area) (HC-030031; n = 9, AITC; n = 9, Saline; n = 10). Prior to administration, significant differences between the groups were not found. In the stand test, the HC-030031 group showed a significantly increased ipsilateral/contralateral ratio compared with the AITC group. No difference between the AITC- and saline-treated groups was found ($F_{2,512} = 66.0$; $p < 0.05$ at each time point comparing HC-030031 with AITC; Fig. 4A). Similar to the stand test, the HC-030031 group showed a significant increase in max contact area compared with the AITC group ($F_{2,512} = 40.6$; $p < 0.05$ at each time point comparing HC-030031 with AITC, and $p < 0.05$ at each time point comparing AITC with saline; Fig. 4B). These results suggest that the inhibition of *Trpa1* activity can reduce OA knee pain.

3.4. PAM comparison of the paw-withdrawal threshold using Trpa1-acting drugs in OA model rats

To detect hypersensitivity in OA knee joints, we used PAM and CatWalk (HC-030031; n = 9, AITC; n = 9, Saline; n = 10). Prior to administration, significant differences between the groups were not found. From 30 min to 24 h after drug administration, the ratio of weight distribution between the hind limbs showed a significant improvement in the pain threshold of the HC-030031 group compared to that of the AITC group. There was no significant difference between the AITC and saline groups except at 3 h ($F_{2,150} = 85.8$; $p < 0.05$ at each time point comparing HC-030031 with AITC, and $p < 0.05$ at each time point comparing saline with AITC; Fig. 4C). These results suggest that TRPA1 in the knee may be activated by mechanical stimulation.

4. Discussion

In our study, we found that *TRPA1* activity plays a role in OA knee pain. *Trpa1* was activated in OA knee joints but not in sham-treated joints. Moreover, inhibition of *Trpa1* activity attenuated pain in knee OA rats in behavioral experiments. Interestingly, we found that there was no difference in the pain threshold between the AITC- and saline-

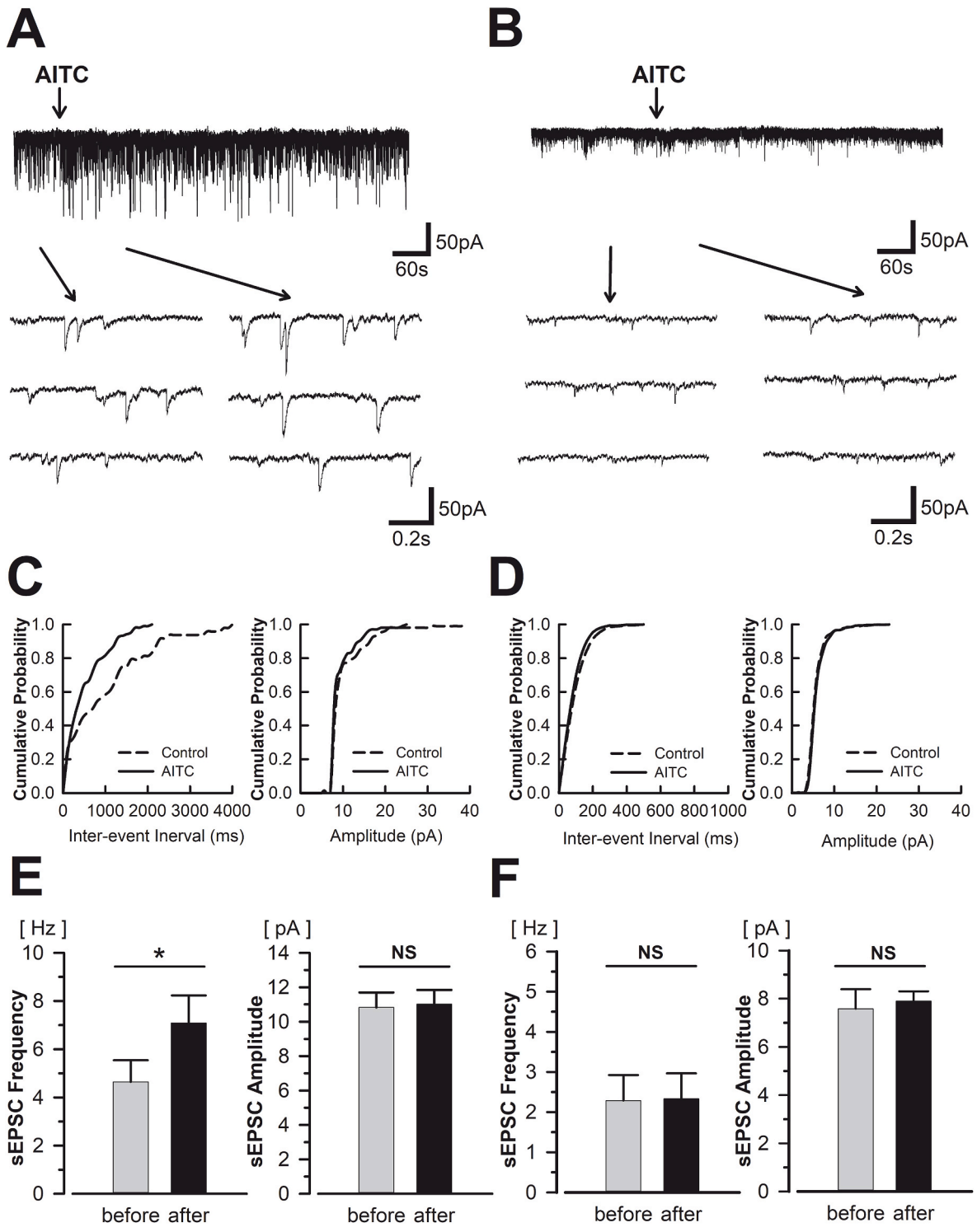

Fig. 2. Effects of allyl isothiocyanate (AITC) on spontaneous excitatory synaptic current (sEPSC) frequency and amplitude in rats with knee osteoarthritis (OA) and Sham (A) A continuous chart recording of glutamatergic sEPSC before and after intra-articular administration of AITC (top) in the knee OA rat. Three consecutive traces of sEPSC are shown in an expanded time scale, before (bottom left) and after the administration of AITC (bottom right). (B) A sham rat. (C) Cumulative distribution of the inter-event interval (left) and amplitude (right) of sEPSC in the control (dotted line) and after the administration of AITC (continuous line; same neuron as in Fig. 2A). (D) A sham rat (same neuron as in Fig. 2B). (E) Summary of sEPSC frequency (left) and amplitude (right) after the intra-articular administration of AITC relative to before the drug administration.in the knee OA rats (n = 7). (F) Sham rats (n = 7). The data are shown as the mean ± SEM. Statistical significance is indicated by an asterisk: *$p < 0.05$. NS: not significant.

treated groups, suggesting that *Trpa1* in the OA knee joint is activated by mechanical stimulation.

4.1. Activation of TRPA1 in the knee OA joint

TRPA1 is expressed in dorsal root ganglia and trigeminal ganglia, especially in nociceptive C and Aδ fibers with small cell body size, and

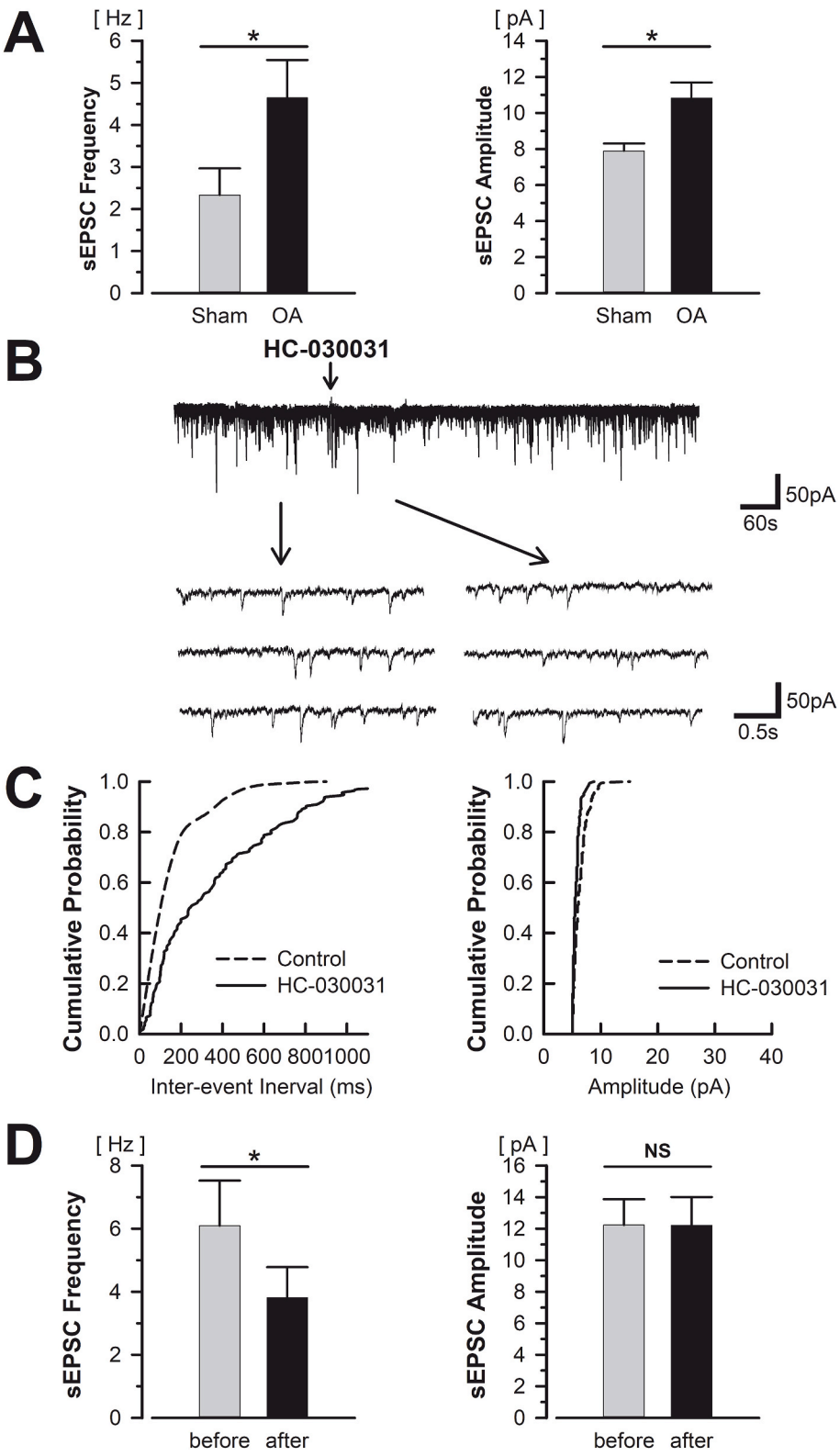

Fig. 3. Effects of transient receptor potential ankyrin 1 (TRPA1) antagonist on spontaneous excitatory synaptic current (sEPSC) in rats with knee osteoarthritis (OA) (A) Summary of sEPSC frequency (left) and amplitude (right) in rats with knee OA (n = 7) before the intra-articular administration of AITC relative to the sham treatment (n = 7). (B) Continuous chart recording of glutamatergic sEPSC before and after intra-articular administration of HC-030031 (top). Three consecutive traces of sEPSC are shown in an expanded time scale, before (bottom left) and after the administration of HC-030031 (bottom right). (C) Cumulative distribution of the inter-event interval (left) and amplitude (right) of sEPSC in control (dotted line) and after the administration of HC-030031 (continuous line; same neuron as in Fig. 3B). (D) Summary of sEPSC frequency (left) and amplitude (right) after the intra-articular administration of HC-030031 (n = 9) relative to before the drug administration. The data are shown as the mean ± SEM. Statistical significance is indicated by an asterisk: $*p < 0.05$. NS: not significant.

responds to intense cold or pungent compounds [9,29]. Although articular cartilage lacks blood vessels and nerves and is considered an inherently painless tissue, many nerve endings are present in the synovial tissue and outer margin of the meniscus of the knee joint. Nerve growth has been reported in these normally uninnervated tissues as a result of angiogenesis, which contributes to pain, and is stimulated by various growth factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and other growth factors [30]. In the present study, *in vivo* patch-clamp confirmed that sEPSC increased in the knee OA group after AITC administration but not in the sham group, suggesting that *Trpa1* activates in the knee OA joint. In addition, reactive oxygen species (ROS) that have been implicated as endogenous agonists of TRPA1 [31,32] have been reported to be associated with the progression of knee OA [33]. We observed an increase in both the frequency

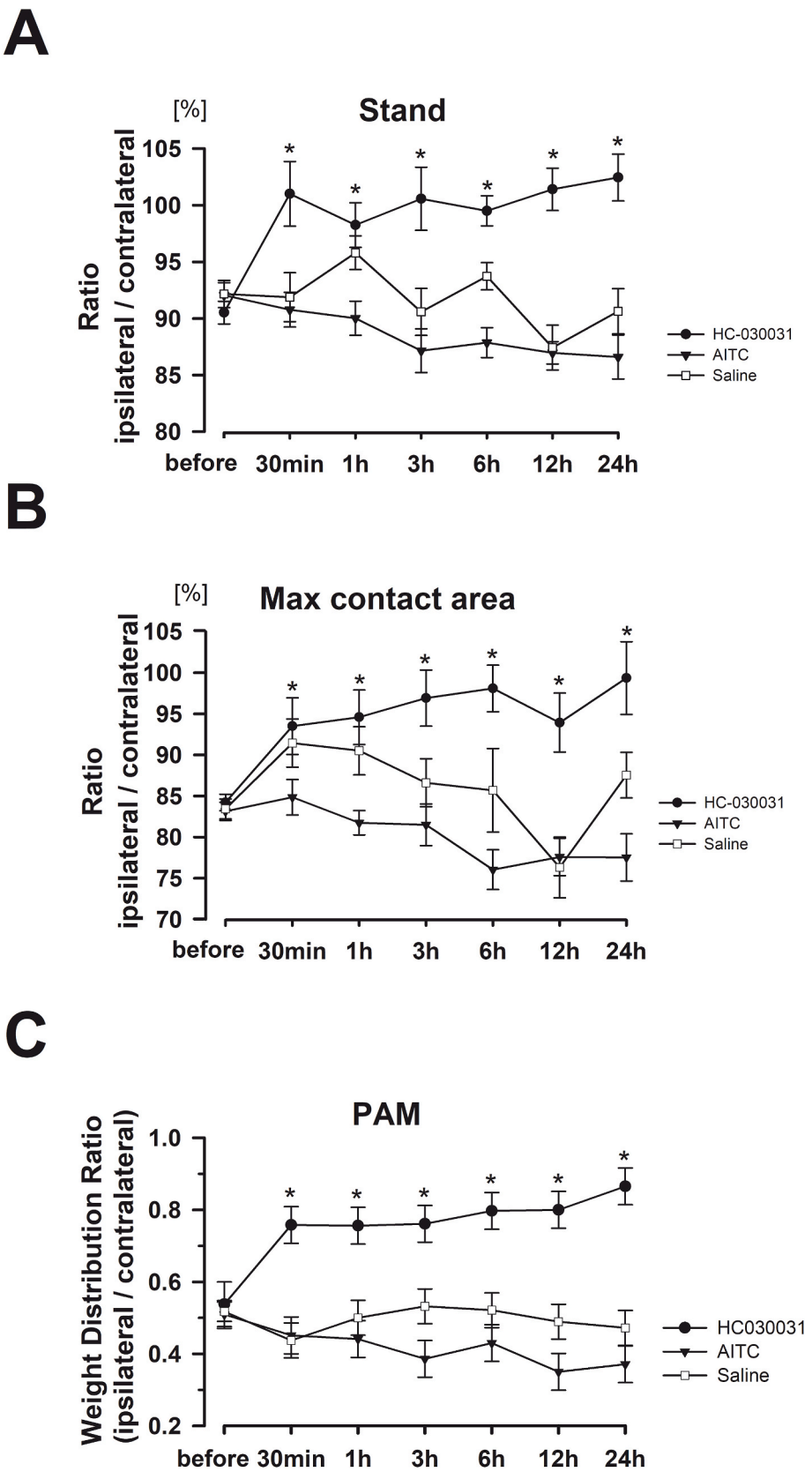

Fig. 4. The results of behavioral tests Circles: HC-030031 (n = 9); triangles: allyl isothiocyanate (AITC) (n = 9); squares: saline (n = 10). (A, B) Individual paw parameters. All data are shown as ratios × 100% between the ipsilateral (right) hind paw and the contralateral (left) hind paw times. (A) The stand data indicates the duration of the stance phase (the period at which the paw is in contact with the glass runway during each step cycle). (B) The max contact area is the size of the paw-print where the print has maximal contact with the glass runway during the stance phase. (C) The ratio of weight distribution in pressure application measurement (PAM) between the hind limbs of the sham-, AITC-, and HC-030031-treated animals. The data are shown as the mean ± SEM. Statistical significance is indicated by $*p < 0.05$.

and amplitude of sEPSC in OA joints before drug administration compared to the sham group. Therefore, we suggest that endogenous agents such as ROS can activate TRPA1 in the OA knee joint.

4.2. TRPA1 activation and mechanical stimulation as an exogenous agonist

It should be noted that the experiments of this study were performed using live animals, but under different conditions between the behavioral and electrophysiological experiments. This suggests the presence of mechanical stress as the synovial cavity is under high mechanical stress, not only in pathological conditions such as OA, but also in normal physiological conditions. The shear force associated with synovial fluid movement promotes oxidative stress, which also acts as endogenous agonists of TRPA1, and affects many cellular functions [34,35]. It has been suggested that TRPA1 plays a role in mechanotransduction in inner ear cells [36], cutaneous sensory neurons [37], and gastrointestinal sensory neurons [38]; however, it has never been reported that mechanical stress activates TRPA1 as an exogenous agonist in joints.

In this study, the PAM results showed that intra-articular administration of HC-030031 improved the threshold of direct pressure stimulation in the rat knee joint compared to AITC. Meanwhile, the CatWalk gait analysis showed similar results. In contrast, neither the PAM nor CatWalk tests demonstrated a significant difference between the administration of AITC and saline. One possible explanation for this is that *Trpa1* is activated, not only by endogenous agonists, but also by mechanical stress, such as load due to walking. This may explain why no significant difference was found between the two groups. In a previous report, the administration of a *Trpa1* inhibitor in a knee OA model reduced mechanical hypersensitivity in nociceptive neurons in the dorsal horn of the spinal cord [20] but did not reduce persistent pain [39].

This study had some limitations. First, we have not directly confirmed where *Trpa1* is localized within the knee joint and how *Trpa1* activates. Second, we have not been able to identify endogenous agonists of *Trpa1*. Third, although we mentioned mechanical stimulation as a potential new agonist of TRPA1 in the current study, we were unable to examine electrophysiology because *in vivo* patch-clamp did not allow pressure stimulation of the knee. Proper in-vivo patch clamp monitoring under load-applied knee conditions would prove that mechanical stimulation acts as an exogenous agonist. We expect that further studies will be conducted to resolve these issues in order to explore the further potential of TRPA1.

5. Conclusion

In this study, we demonstrated that *Trpa1* activated in the knee joints of rats with OA and that *Trpa1* activity enhanced pain caused by knee OA. Contrary to previous reports, our study revealed that *Trpa1* can be activated by mechanical stimulation as a mechanosensor as well as inflammation and plays an essential role in OA knee pain. To the best of our knowledge, this is the first study to indicate that *Trpa1* is activated by mechanical stimulation in the knee joint of OA rats. We expect that this study will help TRPA1 to become a key to a new therapeutic strategy in the treatment of knee OA and contribute to the treatment of degenerative disease.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hidenobu Tamai reports financial support was provided by Japan Society for the Promotion of Science (KAKENHI).

Data availability

Data will be made available on request.

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