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Original Reports

Involvement of ASIC3 and Substance P in Therapeutic Ultrasound–Mediated Analgesia in Mouse Models of Fibromyalgia

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Abstract: **Therapeutic ultrasound (tUS) is widely used in chronic muscle pain control. However, its analgesic molecular mechanism is still not known. Our objective is to reveal the mechanism of the tUS-induced analgesia in mouse models of fibromyalgia. We applied tUS in mice that have developed chronic hyperalgesia induced by intramuscular acidification and determined the tUS frequency at 3 MHz, dosage at 1 W/cm^2 (measured output as 6.3 mW/cm^2) and 100% duty cycle for 3 minutes having the best analgesic effect. Pharmacological and genetic approaches were used to probe the molecular determinants involved in tUS-mediated analgesia. A second mouse model of fibromyalgia induced by intermittent cold stress was further used to validate the mechanism underlying the tUS-mediated analgesia. The tUS-mediated analgesia was abolished by a pre-treatment of NK1 receptor antagonist—RP-67580 or knockout of substance P (*Tac1*$^{-/-}$). Besides, the tUS-mediated analgesia was abolished by ASIC3-selective antagonist APETx2 but not TRPV1-selective antagonist capsazepine, suggesting a role for ASIC3. Moreover, the tUS-mediated analgesia was attenuated by ASIC3-selective nonsteroid anti-inflammation drugs (NSAIDs)—aspirin and diclofenac but not by ASIC1a-selective ibuprofen. We next validated the antinociceptive role of substance P signaling in the model induced by intermittent cold stress, in which tUS-mediated analgesia was abolished in mice lacking *substance P, NK1R, Asic1a, Asic2b,* or *Asic3 gene*. tUS treatment could activate ASIC3-containing channels in muscle afferents to release substance P intramuscularly and exert an analgesic effect in mouse models of fibromyalgia. NSAIDs should be cautiously used or avoided in the tUS treatment.**

Perspective: **Therapeutic ultrasound showed analgesic effects against chronic mechanical hyperalgesia in the mouse model of fibromyalgia through the signaling pathways involving substance P**

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and ASIC3-containing ion channels in muscle afferents. NSAIDs should be cautiously used during tUS treatment.

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Key Words: *Therapeutic ultrasound, substance P, fibromyalgia, acid-sensing ion channel 3*

Chronic widespread muscle pain, characterized by fibromyalgia, is a common and troublesome presentation in pain clinics; however, the current treatment for fibromyalgia remains insufficient.[1] Animal models for fibromyalgia are good tools to test efficacy and mechanism of treatment.[2] Among them, the acid-induced chronic muscle pain model (or the Sluka model) and intermittent cold stress (ICS) models are 2 frequently employed rodent models.[3,4] In the Sluka model, repeated intramuscular acid stimuli and activation of acid-sensing ion channels 1b and 3 (ASIC1b and ASIC3) and transient receptor potential vanilloid 1 (TRPV1) are required to induce chronic hyperalgesia.[5-7] In particular, ASIC3 knockout (*ASIC3*$^{-/-}$) completely abolished the first acid-induced transient hyperalgesia and the development of chronic hyperalgesia induced by a second acid injection.[6] The ICS model can induce chronic hyperalgesia of the hind paws in wild-type and *ASIC3*$^{-/-}$ mice, although *ASIC3* knockout attenuates muscle hyperalgesia.[3] We previously revealed that intramuscular substance P (also known as neurokinin-1, NK1) could inhibit ASIC3 activation in muscle afferent neurons, resulting in an antinociceptive effect in the Sluka model.[6,8] The antinociceptive signal of substance P in muscle afferents acts through the neurokinin receptor 1 (NK1R) in a G-protein-independent manner to open the Kv7, which is very different from its conventional excitatory role as the primary nociceptive transmitter.[9,10] We also reported that low-level laser therapy (LLLT)-mediated analgesia occurred via the activation of TRPV1 and release of substance P in muscle afferents in the Sluka model.[11] The analgesic effect of LLLT can be blocked by pretreatment with the NK1R antagonist RP-67580 and TRPV1 antagonist capsazepine. In addition, no analgesic effect was observed in mice lacking substance P (*Tac1*$^{-/-}$).

Therapeutic ultrasound (tUS) is one of the most widely used physical modalities that reduces chronic pain, such as osteoarthritis, tendinitis, plantar fasciitis, and fibromyalgia.[12] US is a mechanical sound wave absorbed by the target tissue along the penetrating pathway. It is believed that tUS has thermal and nonthermal effects during treatment.[13] The resulting mechanical sonic energy is absorbed by the target tissue and converted into thermal energy, which results in the heating of deeper tissues.[14] These thermal changes translate into various physiological effects, including augmentation of blood flow and capillary permeability, enhancement of fibrous tissue extensibility, elevation of pain threshold, and alteration of neuromuscular activity leading to muscle relaxation. Nonthermal changes, mainly mechanical and acoustic streaming effects of sound waves, also contribute to an increased rate of chemical activity, alteration in the permeability of cell membranes, expansion of gas-filled bubbles, and an increase in fluid flow.[15] However, these effects are not easily separated in real-world practice.[16] It is reasonable to hypothesize that tUS exerts its analgesic effect by stimulating certain types of thermo- and/or mechanoreceptors.

Since tUS has an analgesic role similar to LLLT in chronic muscle pain,[17] we hypothesized that tUS, also having similar analgesic mechanisms like LLLT, could treat chronic muscle pain through the activation of ion channels on muscle afferents and thus modulate intramuscular substance P secretion.[11] This study aimed to elucidate the mechanism underlying tUS analgesia in a mouse model of fibromyalgia.

Methods

Animals

Adult (8–16 weeks old) male (or both male and female in some cases) C57BL/6J mice were used in the Sluka model. Adult (8 weeks old) female C57BL/6J mice were used in the ICS treatment model unless specifically mentioned, since female mice exhibited a longer-lasting mechanical hyperalgesia than male mice exposed to ICS.[18] The animals were obtained from the National Laboratory Animal Center (Taipei, Taiwan). All procedures followed the Guide for the Use of Laboratory Animals (National Academies Press, Washington, DC, USA) and were approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University, and Academia Sinica. Experiments were performed by minimizing the number of mice and their suffering without compromising the quality of the experiments. Mice with tachykinin 1 (*Tac1*$^{-/-}$) were kindly gifted to us from Dr. Zimmer at the University of Bonn[19] and were backcrossed with C57BL/6J mice for > 10 generations. Mice with NK1R knockout (*Tacr1*$^{-/-}$) were generated by Dr. Steve Hunt at University College London, imported from the European Mouse Mutant Archive (EM:05452), and backcrossed with B57BL/6J mice for 10 generations.[20] The congenic *Tac1*$^{-/-}$ and *Tacr1*$^{-/-}$ mice used in this study were offspring of *Tac1*$^{+/-}$ and *Tacr1*$^{+/-}$ intercrosses, respectively, as previously described.[8] *ASIC1a* knockout (*ASIC1a*$^{-/-}$) mice were kindly gifted from Dr. Lien at National Yang Ming Chiao Tung University (Taipei)[21] and the mice used in the behavioral studies were offspring of the heterozygote *ASIC1a*$^{+/-}$ intercross in a congenic C57BL/6J background, as previously described.[22] *Trpv1*$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and generated using *Trpv1*$^{+/-}$ intercrosses. *ASIC1b*$^{-/-}$ and *ASIC3*$^{-/-}$ mice were generated in our laboratory as previously described.[5,23] The experimental design is shown in Table 1.

Generation of ASIC2a and ASIC2b Knockouts

ASIC2a and ASIC2b are encoded by *Accn1*.[22] They differ only in the 5′ terminus of the messenger ribonucleic acid within exon 1. *ASIC2a* and *ASIC2b* knockout

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Table 1. Experimental Design

ANIMALS (C57BL/6J AND SPECIFIC GENE KNOCKOUT MICE)	ANIMAL MODELS	INTERVENTIONS	BEHAVIOR ASSAYS	HISTOLOGY EXAMINATION
Adult male and female	Sluka model (inject at day 0 & 1)Modified Sluka model (inject at day 0 with RP-67580)	Therapeutic US with/ without specific inhibitors injection from day 4 to day 8. (Some followed from day 11 to day 15)	von Frey filament test	pERK expression in dorsal root ganglions
Adult female only	Intermittent cold stress model			

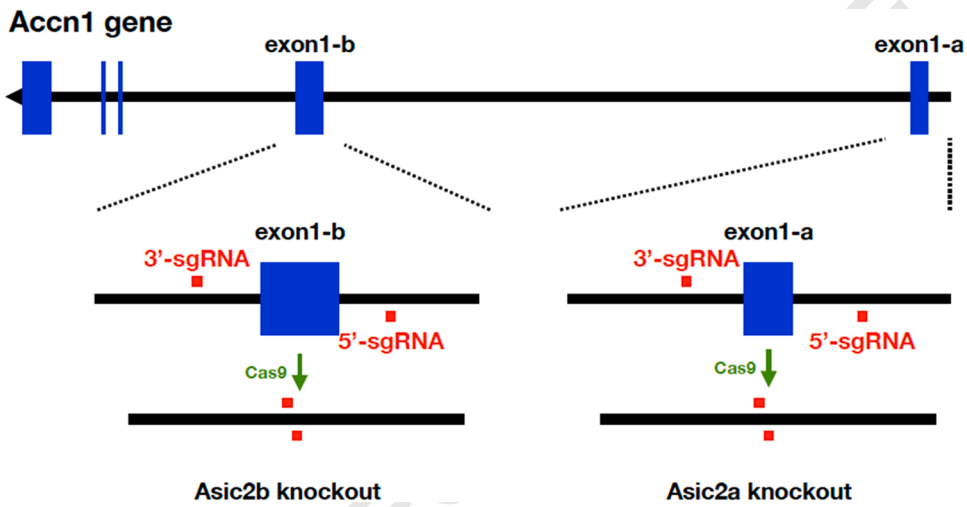

Figure 1. Generation of knockout mice lacking ASIC2a or ASIC2b. Exon composition and experimental design to generate *ASIC2* (*Accn1*) knockout mice using CRISPR/Cas9 technology. To knock down ASIC2a, 2 single-guide RNAs (sgRNAs) were used to target the 5′-upstream and the 3′-downstream of exon 1a of ASIC2, and to knockout ASIC2b, 2 sgRNAs were used to target the 5′-upstream and the 3′-downstream of exon 2b of ASIC2.

(*ASIC2a*$^{-/-}$, *ASIC2b*$^{-/-}$) mouse lines were generated using genome-editing clustered regularly interspaced short palindromic repeat (CRISPR) technology. Briefly, nuclei of C57BL/6J mouse zygotes were injected with Cas9 RNA and 2 single-guide RNAs, one targeting the 5′-upstream region of exon 1 and the other targeting the 3′-downstream region of exon 1 of *ASIC2a* (NM_001034013.2) or *ASIC2b* (NM_001034013.2) (Fig 1). After germline transmission, we screened for the correct exon 1 deletion for *ASIC2a*$^{-/-}$ and *ASIC2b*$^{-/-}$ lines by polymerase chain reaction using the following primers:

ASIC2a-WT-F: GAGAGCTCGGAGAGAGTATCCTAC
ASIC2a-KO-F: TCTACTGTAGGGTCTGCACACATG
ASIC2a-R: TGACAAGATGTTTCTGTCACACG
ASIC2b-WT-F: ACCCCAGTTTTTACGCTGATCC
ASIC2b-WT-R: GTCTCAGCAGAAAGCAGCTCC
ASIC2b-KO-F: GGAAAGACGGGGTAACGGTG
ASIC2b-KO-R: GGAGTTTTCTCCTAGACTCCGTG

Sluka Model of Acid-Induced Chronic Muscle Pain

The Sluka model, a mouse model of noninflammatory chronic muscle pain or fibromyalgia, was used to test the analgesia induced by tUS. In this model, 2 repeated acidic saline injections administered 2 days apart to one side of the gastrocnemius muscle (GM) caused bilateral, long-lasting mechanical hyperalgesia in both hind paws.[7] Acidic saline was prepared in 10 mM 2-[N-morpholino]ethanesulfonic acid and adjusted to pH 4.0 with 1 N NaOH. In this study, the Sluka model was also modified to induce chronic muscle pain using a single intramuscular acid injection combined with an NK1R antagonist, 100 μM 2-[1-imino-2-(2-methoxyphenyl) ethyl]-7,7-diphenyl-4-perhydroisoindolone (3aR, 7aR) (RP-67580, Tocris, Avonmouth, UK), as described.[8] RP-67580 was prepared from a 20 mM stock solution (in 100% ethanol) to a final concentration of 100 μM in saline (pH 7.4). Coinjection of acid saline and RP-67580 can induce long-lasting mechanical hyperalgesia, an exact phenocopy of the Sluka model, and minimize the failure rate of chronic pain induction. Unless specified (while 2 injections of acid saline were applied), we injected 20 μL of acidic saline (pH 4.0) combined with RP-67580 into the GM of the left legs of the mice.

ICS Model

The second mouse model of fibromyalgia used to test the tUS effect was induced by ICS, as established by Ueda et al.[24] In brief, mice were kept in a 4 ± 2 °C (cold

room) overnight from 16:30 to 10:00 the next day, then kept alternatively in rooms at 24 ± 2 °C (room temperature) and 4 ± 2 °C every 30 minutes for 6 hours as a run of ICS treatment. During ICS treatment, food pellets and 1% agar were placed on the mesh floor of the cage. After 2 days of ICS treatment, the mice were housed in individually ventilated cages at room temperature. The mechanical responses of the hind paws and GM were measured before and post-ICS at days 4 to 8.

Behavioral Assays

Animals were housed in a group of 3 to 5 with a 12-hour light/12-h dark cycle (lights on at 8:00). The behavior tests were performed between 9:00 and 16:00 in the light cycle of animals. For behavioral tests, the mice were acclimated to the behavioral rearing room for at least 1 week and transported to the behavioral testing room for at least 1 hour before the tests. Mechanical hyperalgesia was assessed by applying a .2 mN von Frey filament to the plantar surface of both hind paws.[8] A positive response was defined as foot lifting when a von Frey filament was applied. For each paw, the filament was applied 5 times at 30 seconds intervals. Responses of the paw to mechanical stimuli were measured before and 4 hours, 1 day, and 4 days after the acid injection(s) or ICS treatment, as well as daily after tUS.[8] The experimenter who conducted the von Frey test was blinded to the mouse genotypes and/or drug treatments. For the pharmacological experiments, the drug solution was intramuscularly injected into the left GM 3 minutes before tUS treatment. The mice received the solvent intramuscularly as a control. PcTx1, a specific ASIC1a blocker, and capsazepine, a potent TRPV1 antagonist, were purchased from Tocris (Avonmouth, UK).[25] Capsazepine was prepared from a 20 mM stock solution (in 100% ethanol) to a final concentration of 50 μM in saline (pH 7.4). APETx2, a potent and specific ASIC3 inhibitor, was obtained from Alomone Laboratories (Jerusalem, Israel). Aspirin (acetylsalicylic acid), diclofenac, and ibuprofen sodium were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Mouse Models of tUS

TUS was administered to isoflurane (2%) anesthetized animals via the AutoSound 5.6 applicator (Richmar, NY, USA). The ultrasound output was characterized using a hydrophone (HGL-0400, Onda, Sunnyvale, CA, USA), and ultrasound-induced temperature changes were assessed using thermocouples. As the mouse muscle is thin and the ultrasound device produces a 2.5 ms tone burst signal, one should avoid resonance to occur in the muscle. We used a water layer to mimic the muscle layer and found the intensity of 1 MHz stimulation was 16 times higher than 3 MHz. Thus, we only employed 3 MHz in this study. Before tUS treatment, the left leg of each mouse was shaved. Different doses and duty cycles of 3 MHz ultrasound for 3 minutes were applied to the GM daily for 5 days from the fourth day after the acid injection(s) or ICS treatment. In some cases, after the tUS effects disappeared, we employed tUS treatment on the mice again from days 11 to 15 to test the effects of drugs at higher doses or drugs targeting the same ion channels. The responses of both hind paws to mechanical stimuli were measured before and 1.5 hours after tUS. Appropriate receptor antagonists indicated in specific experiments were added to the formula to delineate the molecular pathway. The combined formula was injected intramuscularly into the left GM at the middle level 3 minutes before tUS treatment.

Immunohistochemistry Studies of DRG

We measured extracellular signal-regulated kinase phosphorylation (pERK) as a surrogate marker to assess the tUS-induced neuronal activation of muscle afferent dorsal root ganglia (DRG) neurons. Anesthetized mice received tUS treatment for 3 minutes and were immediately perfused with 3% paraformaldehyde through the femoral vein. Before tUS treatment, the mouse legs were shaved and topically treated with sincaine cream 5% (SINPHAR Pharmaceutical, Ilan, Taiwan) for 10 minutes, which contained lidocaine and prilocaine, to avoid contact-induced pERK expression in cutaneous afferent DRG neurons. Lumbar 4 DRGs were collected, postfixed, dehydrated with 30% sucrose for 1 day, and then embedded in optimal cutting temperature compound for frozen sections. DRG sections were cut to 12 μm thickness using a Leica CM3050S cryostat and incubated with blocking solution (50 mmol/L Tris [hydroxymethyl]aminomethane-buffered saline containing 1% bovine serum albumin, .1% Triton X-100, and .002% sodium azide) for 2 hours. Primary antibodies against pERK (dilution 1:500; Cell Signaling Technology, Boston, MA, USA) and substance P (dilution 1:500; Neuromics, Northfield, MN) were diluted in the blocking solution. The secondary antibodies used were Alexa Fluor 488(ab′)2 fragments of goat antirabbit, and Alexa Fluor 594(ab′)2 fragments of goat antiguinea pig (1:1000 in blocking solution, Molecular Probes, Thermo Fisher Scientific, Waltham, MA). Images were acquired using a confocal microscope (Zeiss LSM700 Stage; Zeiss, Oberkochen, Germany).

Statistical Analysis

GraphPad Prism 7.0 (GraphPad, San Diego, CA) was used for statistical analyses, and all results are presented as the means ± SEM. Behavioral data were analyzed using a 2-way analysis of variance followed by the posthoc Holm-Sidak test. pERK data were analyzed using the Student's t-test. $P < .05$ was considered statistically significant.

Results

Dose-Dependent Effects of tUS on Acid-Induced Mechanical Hyperalgesia in the Sluka Model

The tUS treatment was delivered via a 2 cm^2 "Therapy Hammer" transducer to the left GM for 3 minutes (Fig 2A). Compared with sham treatment, unilateral

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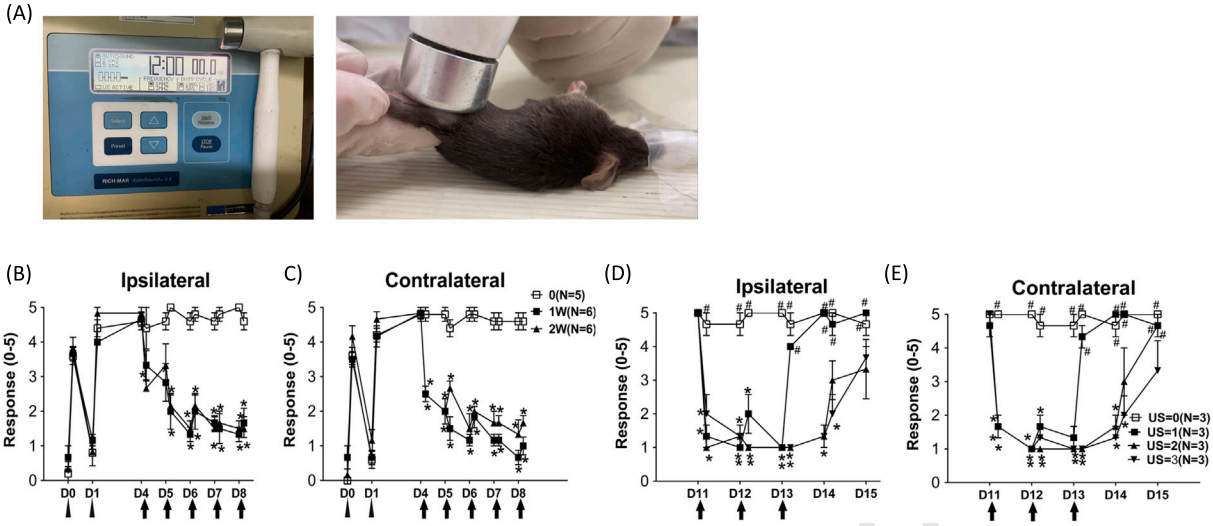

Figure 2. Analgesic effect of different doses of ultrasound on acid-induced chronic mechanical hyperalgesia. (**A**) Setup of the therapeutic ultrasound treatment. The mouse was anesthetized with 2% isoflurane. The ultrasound treatment was applied to the surface of the gastrocnemius muscle for 3 minutes. (**B**, **C**) Two intramuscular injections of acidic saline (pH 4.0) on day 0 and day 1, respectively, were used to induce bilateral chronic mechanical hyperalgesia of the hind paws in the mice. From days 4 to 8, the mice received different doses daily (0, 1, and 2 W/cm^2) of 3 MHz ultrasound treatment (100% duty cycles) for 3 minutes. (**D**, **E**) The mice with acid-induced chronic hyperalgesia were treated with ultrasound daily for 1, 2, or 3 days or without ultrasound. The withdrawal responses of the hind paws were evaluated before and 1.5 hours after the ultrasound treatment. The arrowheads indicate the dates with intramuscular acid injection. The arrows indicate the ultrasound treatment. D, day; US, ultrasound stimulation. *P < .05, **P < .01 compared with days 4 or 11 before ultrasound treatment; #P < .05 compared with D0.

treatment with 3 MHz tUS (100% duty cycles) at 1 and 2 W/cm^2 (based on device setting) significantly reduced mechanical hyperalgesia on both the ipsilateral and contralateral hind paws of wild-type mice that received 2 intramuscular acid injections (Figs 2B and 2C). The analgesic effects of tUS lasted for 24 to 48 hours (Figs 2D and 2E) and were not compromised by repeated daily treatment for 5 days (Figs 2B and 2C). Since the device setting at 1 W/cm^2 was adequate to produce an analgesic effect on chronic muscle pain, we chose 1 W/cm^2 as our treatment dose in subsequent experiments.

Next, we investigated the effect of duty cycles on tUS analgesia. Mice with chronic hyperalgesia were treated with 3 MHz and 1 W/cm^2 of tUS at different duty cycles (20, 50, and 100%) for 3 minutes on the GM surface of the left leg. Compared with pre-tUS, unilateral tUS treatment with 50% and 100% duty cycles significantly reduced acid-induced hyperalgesia in both the ipsilateral and contralateral hind paws (Fig 3A). Accordingly, we chose a device setting of 3 MHz, 100% duty cycle, and dosage of 1 W/cm^2 as our treatment dose in later experiments, as it showed the best analgesic effect in the Sluka model. To determine the ultrasound intensity, the ultrasound probe output was characterized using a hydrophone. A culture dish was placed on top of the probe to create a water buffer that represented the muscle layer (Fig 3B). The measured ultrasound amplitude across the probe showed an uneven distribution with multiple hotspots (Fig 3C). Using this setup, we measured the ultrasound intensity and peak stress around the center of the probe at different duty cycles (Fig 3D). In general, the measured peak stress remained the same in all duty cycles, whereas the intensity increased with the duty cycles. The ultrasound output at a 100% duty cycle was still very low, with an intensity of 6.3 mW/cm^2 and a peak stress of 16.2 kPa. The temperature increase in water was less than 1.5 °C in 3 minutes, suggesting a minimum thermal effect in our animal studies (Fig 3E).

RP-67580 and Tac1 Knockout Inhibited the tUS Analgesia in the Sluka Model

We have previously shown that substance P signaling is antinociceptive in muscle afferents in the Sluka model of chronic muscle pain.[5] To test if substance P signaling is involved in tUS-mediated analgesia, we blocked the substance P receptor, NK1R, 3 minutes before tUS treatment. Intramuscular injection of RP-67580, a potent and selective tachykinin NK1R antagonist, abolished tUS-induced analgesia in the Sluka model, whereas tUS treatment significantly reduced acid-induced mechanical hyperalgesia in the vehicle group (Figs 4A and 4B).

The tachykinin 1 gene encodes 4 products of the tachykinin peptide hormone family, substance P, and neurokinin A, as well as related peptides, neuropeptide K and neuropeptide gamma. We chose *Tac1*$^{-/-}$ mice to further validate the involvement of substance P in tUS analgesia. Similar to the results of RP-67580, tUS significantly reduced acid-induced mechanical hyperalgesia in wild-type mice but had no effect on *Tac1*$^{-/-}$ mice (Figs 4C and 4D). These results suggest an analgesic role for substance P in tUS treatment in the Sluka model of chronic widespread pain.

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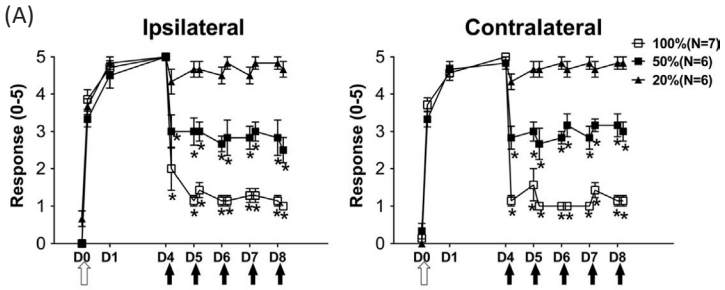

(D)

Ultrasound data

Duty cycle	Intensity (mW/cm^2)	Peak stress (kPa)
100%	6.3	16.2
50%	1.8	13.3
20%	1.4	16.7

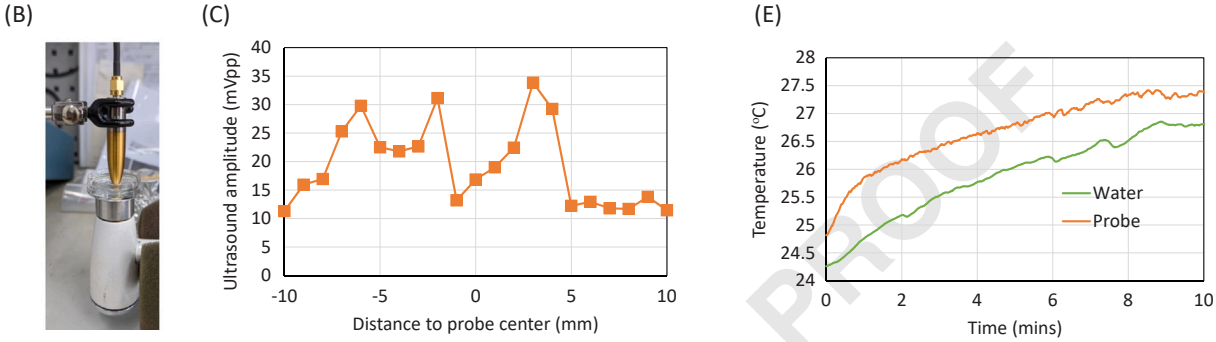

Figure 3. Effects of duty cycles on ultrasound output and ultrasound-mediated analgesia. (**A**) When coinjection with 100 μM RP-67580 (on day 0), a single intramuscular acid injection (pH 4.0), was sufficient for the development of bilateral chronic mechanical hyperalgesia of the hind paws in mice. On days 4 to 8, the mice received different duty cycles daily of 1 W/cm^2 ultrasound at a frequency of 3 MHz for 3 minutes. The withdrawal responses were evaluated before and 1.5 hours after the ultrasound treatment. Blank arrows indicate the i.m. acid injection combined with RP-67580. Filled arrows indicate the ultrasound treatment. D, day. *$P < .05$, **$P < .01$ compared with day 4 before ultrasound treatment. (**B**) The measurement setup of ultrasound output. The hydrophone was immersed in the water of a culture dish, which was placed on top of the ultrasound probe. (**C**) Ultrasound peak stress (kPa) measured across the probe. (**D**) The measured ultrasound intensities and peak stress at different duty cycles. (**E**) The measured temperature increased with time during the ultrasound stimulation.

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Involvement of ASIC3 in tUS Analgesia

Next, we probed the molecular determinants that responded to tUS stimulation, which led to substance P release. Because tUS might produce thermal, mechanical, and acoustic streaming effects, we tested the roles of thermal transducers (TRPV1 channels) and acoustic streaming transducers (ASICs) in tUS analgesia.[26,27]

We first tested the role of TRPV1 in tUS analgesia because TRPV1 functions as a pain and temperature sensor in mammals.[28] We have previously shown that capsazepine, a selective antagonist of TRPV1, can abolish LLLT-mediated analgesia in the Sluka model.[11] Mice preinjected with capsazepine had the same levels of mechanical hyperalgesia after tUS as those in the control group, which received only tUS without capsazepine injection. This phenomenon was noted in both the ipsilateral and contralateral paws (Figs 5A and 5B). Thus, tUS-induced analgesia does not occur through the TRPV1 pathway.

We next examined the roles of ASIC subtypes in tUS analgesia because ASICs are known to be dual-function proteins involved in both acid-sensing and mechano-sensing.[29] The mechano-sensing properties of ASICs are specified in tether-mode mechanotransduction, in which mechanically sensitive ion channels are opened via stretching of force-transducing proteins that tether to the ion channels from the extracellular matrix and intracellular cytoskeleton proteins.[23,30]

Currently, selective antagonists for ASIC subtypes include PcTx1, a 40-amino acid toxin from tarantula venom targeting ASIC1a[31]; mambalgin-1, a 57-amino acid toxin from mamba snake venom targeting ASIC1a and ASIC1b[32]; and APETx2, a 42-amino acid toxin isolated from the sea anemone *Anthopleura elegantissima* targeting ASIC3.[33] Pretreatment with PcTx1 and mambalgin-1 had no effect on tUS-mediated analgesia in the Sluka model (Figs 5C-5F), whereas APETx2 abolished tUS analgesia (Figs 5G and 5H). These results are intriguing because ASIC1a could be mechanically activated to mediate an antinociceptive effect via the release of substance P in dextrose prolotherapy models.[34] Therefore, we further validated the effect of tUS in *ASIC1a*$^{-/-}$ mice. Consistently, tUS significantly reduced acid-induced mechanical hyperalgesia in both male and female *ASIC1a*$^{-/-}$ mice (Figs 6A-6D). Thus, tUS-induced analgesia is mediated through an ASIC3-dependent pathway.

Aspirin and Diclofenac, but Not Ibuprofen, Diminished the tUS-Mediated Analgesia

We further validated the role of ASIC3 in tUS analgesia. Since intramuscular acidification induces non-inflammatory muscle pain in the Sluka model, we can validate the roles of ASICs in tUS analgesia with non-steroidal anti-inflammatory drugs (NSAIDs) that show

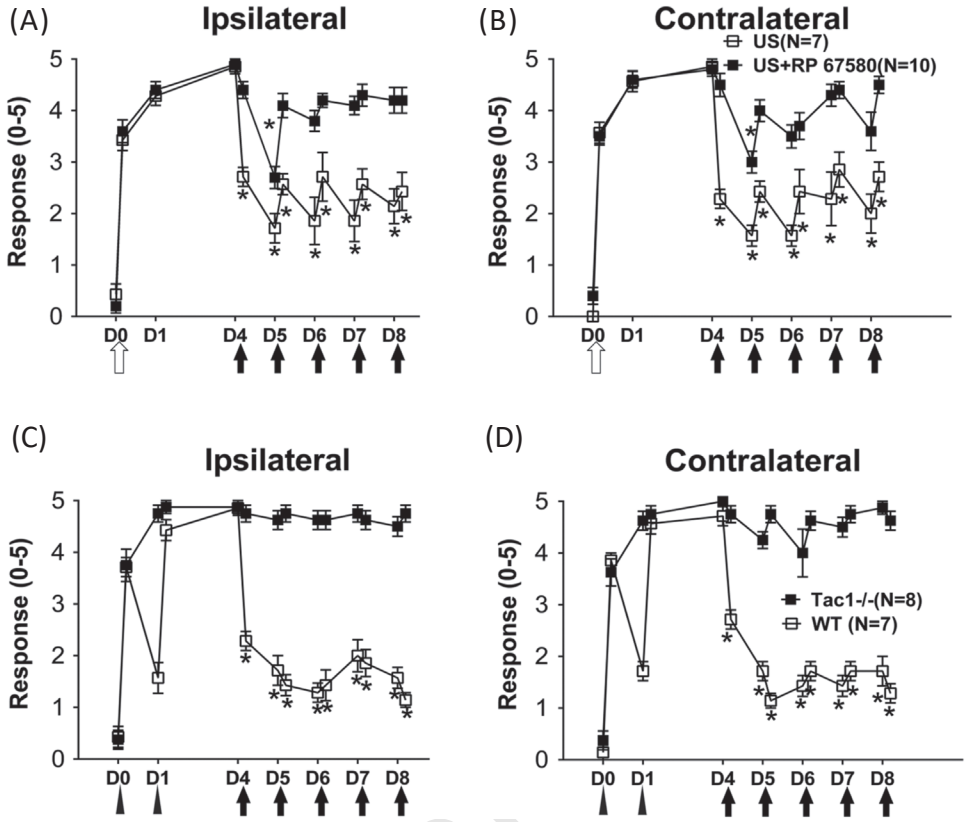

Figure 4. Involvement of substance P in ultrasound-mediated analgesia. (**A**, **B**) The analgesic effect of ultrasound treatment was tested in mice with chronic mechanical hyperalgesia induced by a single intramuscular acid injection combined with 100 μM RP-67580 (on day 0). From days 4 to 8, the mice received daily saline (N = 7) or 100 μM RP-67580 (N = 10) injection 3 minutes before a 3 minutes ultrasound treatment at the condition of 1 W/cm^2, 3 MHz. (**C**, **D**) A single intramuscular injection of acidic saline (pH 4.0) on day 0 in substance P knockout (*Tac1*$^{-/-}$, N = 8) mice or 2 injections on days 0 and 1 in wild-type mice (WT, N = 7) were used to induce bilateral chronic mechanical hyperalgesia of the hind paws. From days 4 to 8, the mice received 1 W/cm^2 ultrasound treatment daily at a frequency of 3 MHz for 3 minutes at the ipsilateral gastrocnemius muscle. The withdrawal tests were evaluated before and 1.5 hours after the treatment. The analgesic effect of ultrasound was abolished in the RP-67580 injection and *Tac1*$^{-/-}$ mice group. The blank arrows and filled arrowheads indicate the intramuscular acid injection with or without RP-67580, respectively. The filled arrows indicate the time points with ultrasound treatment. D, day. **P* < .05 compared with day 4 before the ultrasound treatment.

selective inhibition of ASIC subtypes. Aspirin and diclofenac reduce inflammation and pain in clinical conditions and specifically inhibit ASIC3; ibuprofen, another NSAID used primarily to treat fever and pain, also selectively inhibits ASIC1a.[35]

Mice preinjected with 20 μL of 500 μM and 5 mM aspirin or 200 μM diclofenac in the GM before tUS partially reduced mechanical hyperalgesia but compromised the tUS analgesic effect. With tUS treatment, the mice preinjected with aspirin and diclofenac showed more severe pain-like responses than those preinjected with saline (Figs 7A-7D). However, tUS analgesia was not affected in mice preinjected with 20 μL of 500 μM ibuprofen in the GM (Figs 7E and 7F). Together, these results indicate that aspirin and diclofenac can inhibit the analgesic effect of tUS, suggesting an ASIC3-dependent pathway for tUS analgesia.

Molecular Determinants of tUS-Mediated Analgesia in a Mouse Model of ICS-Induced Fibromyalgia

Since intramuscular acid injection-induced mechanical hyperalgesia depends on the activation of ASIC1b, ASIC3, and TRPV1, we had no way to validate the tUS analgesia in the corresponding knockout mice in the Sluka model. In particular, ASIC3-dependent pain chronicity and ASIC3-mediated analgesia are paradoxical. Thus, we adopted a second mouse model of fibromyalgia induced by ICS, in which ICS-treated mice developed mechanical hyperalgesia of the hind paws independent of ASIC3.[3] Thus, we successfully induced widespread mechanical hyperalgesia in the ICS model in mice lacking substance P, NK1R, ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, or TRPV1 (Fig 8). tUS treatment showed analgesic effects in wild-type mice with ICS-induced chronic mechanical hyperalgesia. As expected, the analgesic effect of tUS was completely abolished in *Tac1*$^{-/-}$*and Tacr1*$^{-/-}$ mice (Figs 8A and 8B). tUS analgesia was also abolished in *ASIC1a*$^{-/-}$, *ASIC2b*$^{-/-}$, and *ASIC3*$^{-/-}$ mice but was not affected in *ASIC1b*$^{-/-}$, *ASIC2a*$^{-/-}$, and *Trpv1*$^{-/-}$ mice treated with ICS (Figs 8C-8H). Intriguingly, tUS analgesia was induced in the first trial but was abolished in consecutive trials in *ASIC1a*$^{-/-}$ mice (Fig 8C). Together, we revealed that substance P and NK1R, as well as ASIC1a, ASIC2b, and ASIC3, were involved in tUS-mediated analgesia in the ICS model.

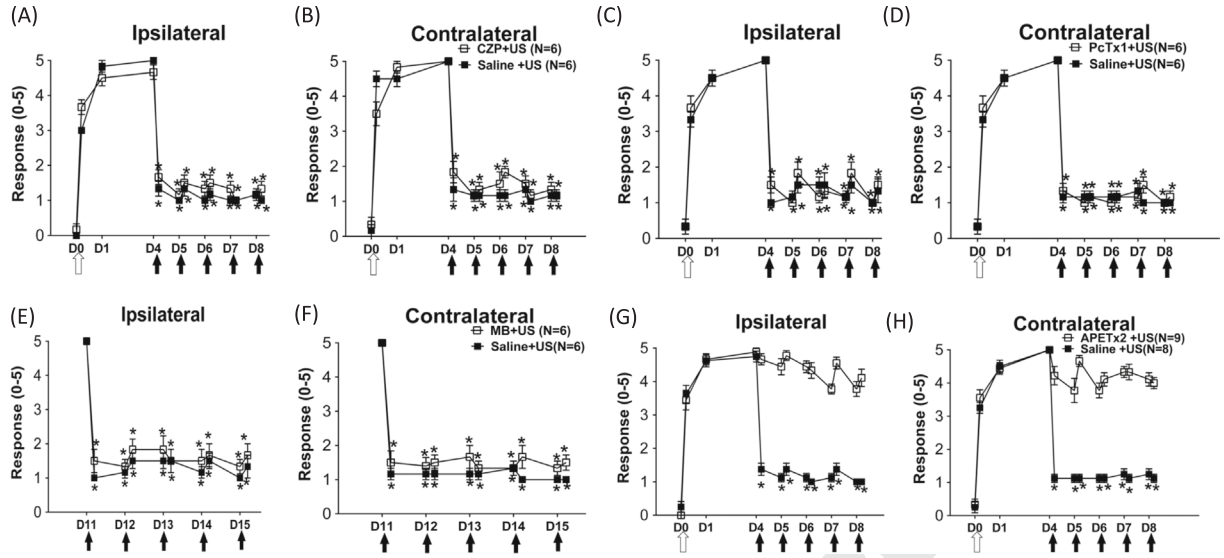

Figure 5. Roles of ASIC1a, ASIC1b, ASIC3, and TRPV1 in ultrasound-mediated analgesia. The effects of selective ion channel blockers on ultrasound-mediated analgesia were tested in the mice with chronic mechanical hyperalgesia induced by a single intramuscular acid injection combined with 100 μM RP-67580 (on day 0). From days 4 to 8 after the acid injection, the mice received a daily injection of 20 μL saline, (**A**, **B**) 50 M capsazepine, (**C**, **D**) 10 nM PcTx1, (**E**, **F**) mambalgin-1 (MB1), or (**G**, **H**) 40 nM APETx2 3 minutes before a 3 minutes ultrasound treatment at 1 W/cm^2 and 3 MHz. The withdrawal tests were performed before and 1.5 hours after the treatment. The blank arrows indicate the intramuscular acid injection of RP-67580. The filled arrows indicate the time points of ultrasound treatment. D, day. *P < .05, compared with day 4 before ultrasound treatment.

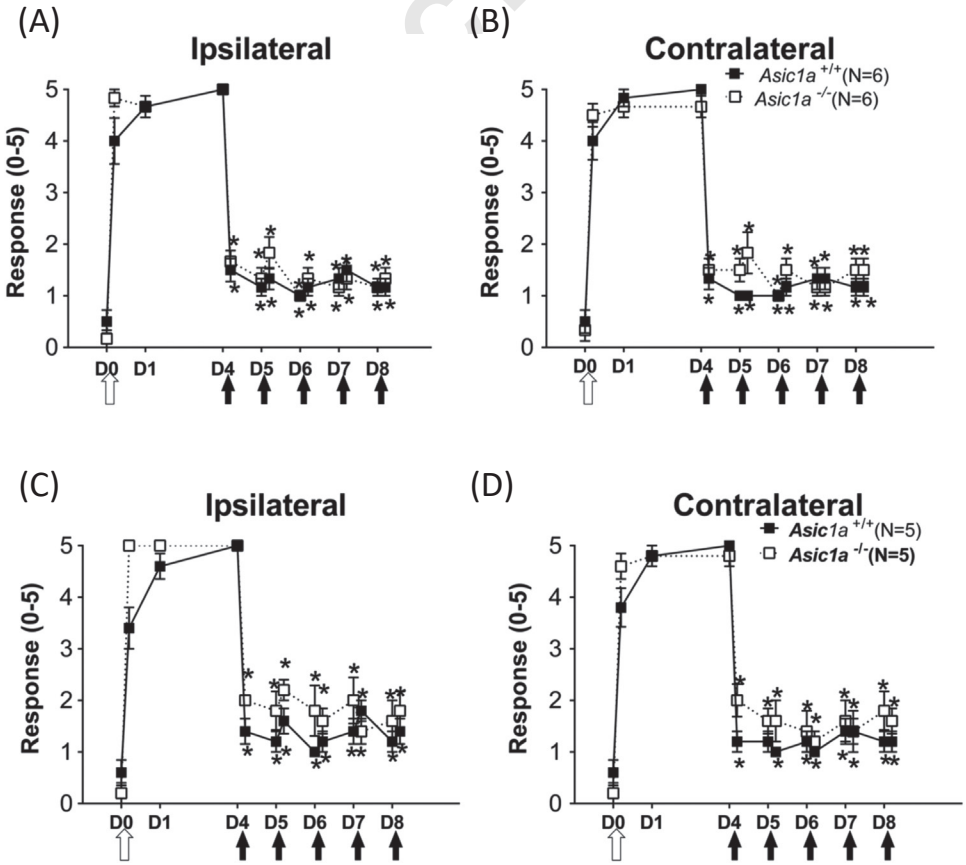

Figure 6. Effect of ultrasound-mediated analgesia in *ASIC1a* knockout mice. The analgesic effect of ultrasound treatment was tested in mice with chronic mechanical hyperalgesia induced by a single intramuscular acid injection combined with 100 μM RP-67580 (on day 0). Starting on day 4, the mice received daily ultrasound (US) treatment at the condition of 1 W/cm^2, 3 MHz for 3 minutes in the gastrocnemius muscle for 5 days. (**A**, **B**) The effect of ultrasound on male *ASIC1a*$^{+/+}$ and *ASIC1a*$^{-/-}$ mice. (**C**, **D**) Effect of ultrasound on female *ASIC1a*$^{+/+}$ and *ASIC1a*$^{-/-}$ mice. The blank arrows indicate intramuscular acid injection with RP-67580. The filled arrows indicate the time points of ultrasound treatment. D, day. *P < .05, compared with day 4 before ultrasound treatment.

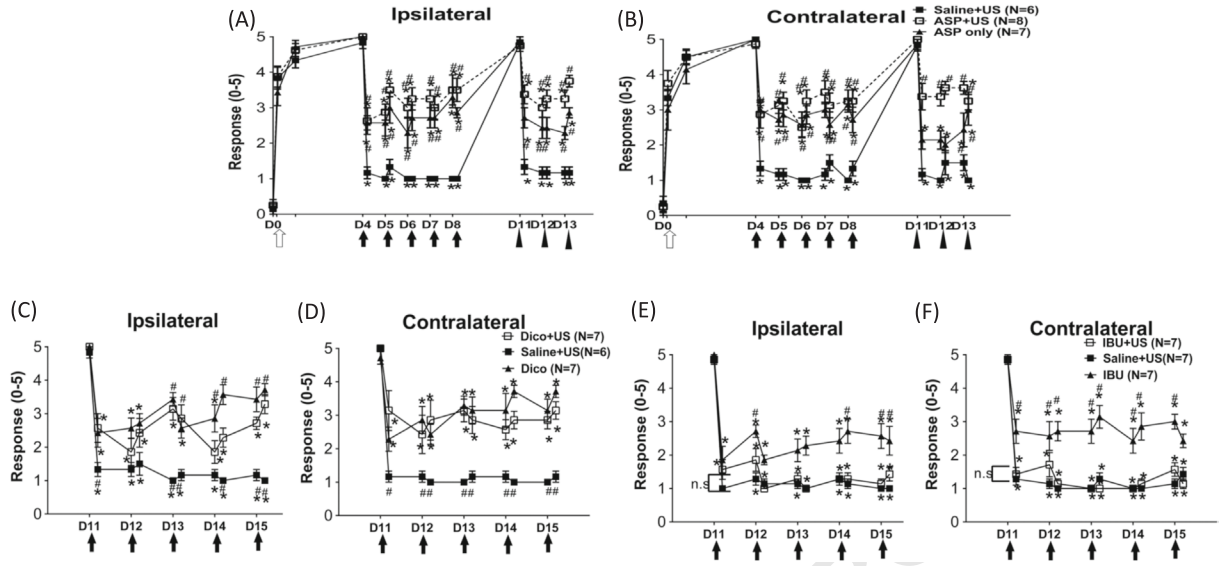

Figure 7. Effect of aspirin, diclofenac, and ibuprofen on ultrasound-mediated analgesia. The effects of NSAIDs on ultrasound-mediated analgesia were tested in mice with chronic mechanical hyperalgesia induced by a single intramuscular acid injection combined with 100 μM RP-67580. Mice received daily injections of saline or (**A**, **B**) aspirin (ASP) in 500 μM (filled arrows) or 5 mM (filled arrowheads), (**C**, **D**) 200 μM diclofenac (Dico), or (**E**, **F**) 500 μM ibuprofen (IBU) 3 minutes before a 3 minutes ultrasound (US) treatment at 1 W/cm^2 and 3 MHz. The withdrawal tests were performed before and 1.5 hours after the treatment. The blank arrows indicate intramuscular acid injections combined with RP-67580. The filled arrows or arrowheads indicate the time points of ultrasound treatment. D, day. *P < .05, compared to the day before the injection and ultrasound treatment. #P < .05, compared with the saline + US group on the same day.

US-Induced pERK Expression in Substance P-Expressing DRG Neurons

To further understand how tUS can mediate an antinociceptive effect, we examined whether tUS could selectively activate substance P-expressing DRG neurons in an ASIC3-dependent manner. Since the antinociceptive role of substance P signal is specific for muscle afferent neurons, we carefully examined tUS-induced pERK expression by blocking the activation of skin afferent neurons with local anesthetics (lidocaine and prilocaine). As a result, tUS only induced pERK expression in a small portion of DRG neurons, mainly colocalized with substance P expression, and the tUS-induced pERK expression was significantly reduced in *ASIC3*$^{-/-}$ mice (Figs 9A-9F).

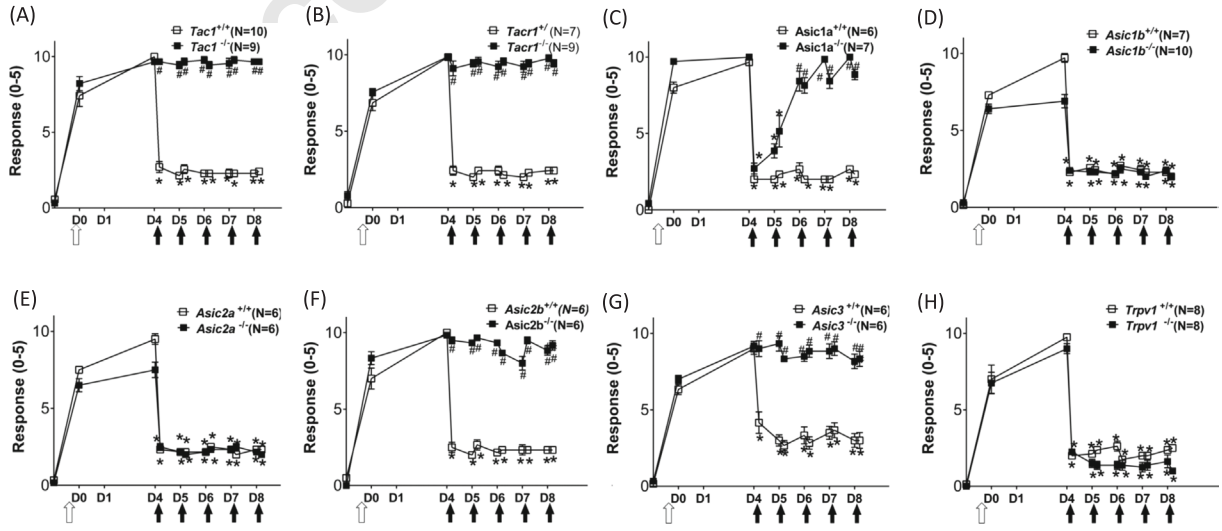

Figure 8. Analgesic effect of tUS on ICS-induced chronic mechanical hyperalgesia in *Tac1*$^{-/-}$, *Tacr1*$^{-/-}$, *ASIC1a*$^{-/-}$, *ASIC1b*$^{-/-}$, *ASIC2a*$^{-/-}$, *ASIC2b*$^{-/-}$, *ASIC3*$^{-/-}$, *and Trpv1*$^{-/-}$ mice. The analgesic effects of tUS were tested in mice with ICS-induced chronic hyperalgesia. On day 4 after the ICS treatment, (**A**) wild-type (WT) mice (N = 10) and *Tac1*$^{-/-}$ mice (N = 9), (**B**) WT mice (N = 7), and *Tacr1*$^{-/-}$ mice (N = 9), (**C**) WT mice (N = 6) and *ASIC1a*$^{-/-}$ mice (N = 7), (**D**) WT mice (N = 7) and *ASIC1b*$^{-/-}$ mice (N = 10), (**E**) WT mice (N = 6) and *ASIC2a*$^{-/-}$ mice (N = 6), (**F**) WT mice (N = 6) and *ASIC2b*$^{-/-}$ mice (N = 6), (**G**) WT mice (N = 6) and *ASIC3*$^{-/-}$ mice (N = 6), and (**H**) WT mice (N = 8) and *Trpv1*$^{-/-}$ mice (N = 8) received daily ultrasound (US) treatment at the condition of 1 W/cm^2, 3 MHz for 3 minutes at the gastrocnemius muscle for 5 days. The blank arrows indicate the intramuscular acid injections combined with RP-67580. The filled arrows indicate the time points of ultrasound treatment. D, day. *P < .05, compared with day 4 before tUS; #P < .05, compared with WT.

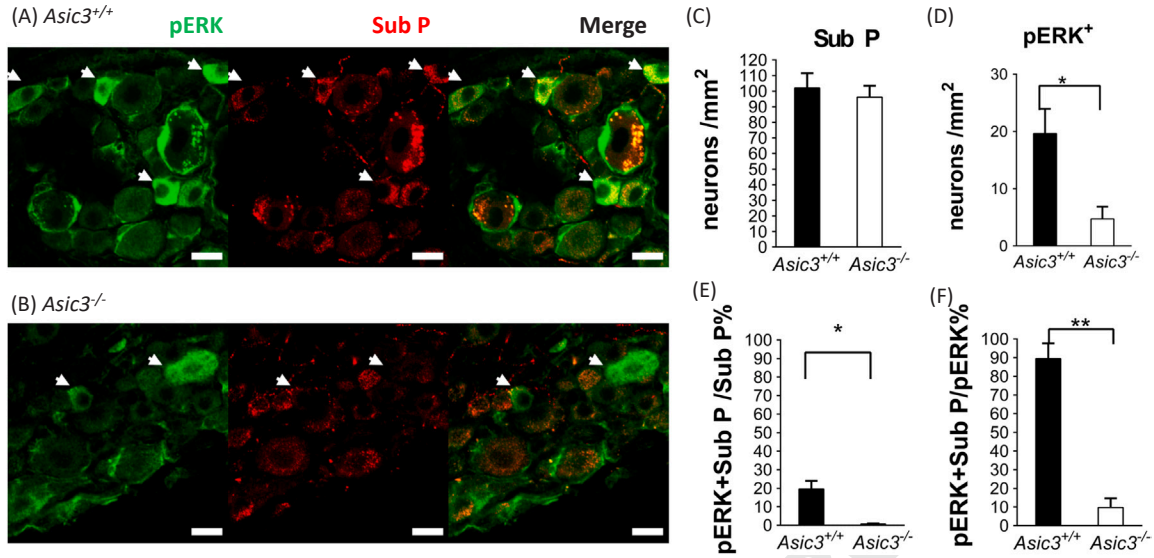

Figure 9. Involvement of acid-sensing ion channel 3-dependent pERK expression in tUS analgesia. (**A**) Representative images of the dorsal root ganglia (DRG) sections showing tUS-induced pERK expression in substance P-positive neurons in wild-type (*ASIC3*$^{+/+}$) mice. (**B**) Representative images of tUS-induced pERK expression in DRG sections of ASIC3 knockout (*ASIC3*$^{-/-}$) mice. (**C**) Quantitative analyses of substance P expression in the DRGs of *ASIC3*$^{+/+}$ and *ASIC3*$^{-/-}$ mice. (**D**) Quantitative analyses of pERK expression in the DRGs of *ASIC3*$^{+/+}$ and *ASIC3*$^{-/-}$ mice. (**E**) Quantitative analyses of pERK expression in substance P-expressing DRG neurons. (**F**) Ratios of pERK-expressing DRG neurons colocalized with substance P. The arrows indicate pERK immunoreactive neurons. *P < .05, **P < .01 between genotypes, scale bar = 20 μm.

Conclusions

Here, we successfully demonstrated that tUS analgesia can be achieved via low-intensity ultrasound-mediated mechanotransduction with minimal thermal effects in mouse models of fibromyalgia induced by intramuscular acid injection(s) (the Sluka model) and ICS (ICS model) and revealed that the mechanism underlying tUS analgesia was mediated via the ASIC3-substance P signaling pathway. Low-intensity ultrasound with a dose of 1 W/cm^2 (measured ultrasound output of 6.3 mW/cm^2) showed analgesic effects lasting for over 24 hours in mice, and repeated daily ultrasound treatment was not found to compromise the analgesic effect. The 100% duty cycle showed the best analgesic effect when 3 MHz ultrasound was applied for 3 minutes in the Sluka model. In addition, the same tUS condition mediated the analgesic effect in the ICS model. Substance P and ASIC3 are involved in tUS-induced analgesia, according to 5 pieces of evidence. First, pretreatment with a potent tachykinin antagonist can block tUS analgesia. Second, tUS has no analgesic effect in *Tac1*$^{-/-}$ and *Tacr1*$^{-/-}$ mice, both of which have impaired substance P signaling. Third, the analgesic effect of tUS can be inhibited by ASIC3 inhibitors, including APETx2, aspirin, and diclofenac, in the Sluka model. Fourth, the analgesic effect of tUS was abolished in *ASIC3*$^{-/-}$ mice in the ICS model. Fifth, tUS treatment selectively activated pERK expression in substance P-expressing DRG neurons of wild-type mice, but pERK-responsive neurons were largely reduced in *ASIC3*$^{-/-}$ mice.

Intriguingly, tUS analgesia is mediated via the same antinociceptive pathway of substance P signaling in muscle afferents, as shown in laser therapy, prolotherapy, and acid-mediated antinociception.[9,11,34,36] Substance P is generally known as a neurotransmitter that conveys pain signals from nociceptors in many pain models.[37] However, accumulated evidence has shown that intramuscular injection of substance P does not generate pain.[38] We previously demonstrated that substance P acts on NK1Rs via a G-protein-independent, tyrosine kinase-dependent pathway in muscle afferents, which enhances M-type potassium currents to silence nociceptor excitation.[8] In addition, substance P can also trigger the release of reactive oxygen species to augment M-type potassium currents, thereby suppressing the excitability of nociceptors.[39] Therefore, how tUS triggers the release of substance P from muscle afferents to treat chronic muscle pain is an interesting topic for further investigation.

Ultrasound is not only applied to musculoskeletal pain control in the field of physical medicine but also in the modulation of certain brain regions for the treatment of neuropsychiatric diseases.[40] Similar to US neuromodulation, the potential mechanisms of US-induced analgesia include mechanosensitive ion channel modulation, membrane deformation, intramembrane cavitation model, and thermal modulation.[41] The cavitation phenomenon is less likely in our study because it usually occurs at submegahertz frequencies.[42] Several thermo- and mechanosensitive ion channels have been proposed as receptors of the US, including TRPV1, TREK-1/2, MEC-4, and ASICs.[27,43-46] A recent study showed that the application of 1.7 MHz focused ultrasound (1.0 MPa, 10 Hz pulse repetition frequency, duty cycles 40%) for 30 seconds on cultured cells in vitro or the mouse brain in vivo could increase the local temperature from 37 °C

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to 42 °C and thus activate TRPV1, which is a thermosensitive ion channel responding to noxious heat.[46] However, we did not observe an essential role of TRPV1 in our tUS condition because neither the TRPV1 antagonist capsazepine nor *Trpv1* knockout abolished tUS analgesia in the mouse models of fibromyalgia. This was consistent with our probe output characterization (Fig 3E), suggesting a limited thermal effect, if any, in our setup. In contrast, the inhibitory effects of ASIC3 antagonists (APETx2, aspirin, and diclofenac) and *ASIC3* knockout on tUS analgesia suggest that tUS might trigger tether-mode mechanotransduction in the muscle.

ASIC3, which belongs to the epithelial Na+ channel/degenerin/acid-sensing ion channel (ENaC/DEG/ASIC) gene family, is a dual-function protein involved in acid sensation and mechanosensation.[29,47] ASIC3 is one of the most sensitive acid sensors in the somatosensory nerve systems.[48] With high sensitivity to acidosis, ASIC3 is involved in pain-associated tissue acidosis occurring in ischemia, inflammation, and fatiguing exercise.[49] ASIC3, together with ASIC1b and TRPV1, are essential acid sensors for acid-induced pain chronicity in the Sluka model.[5-7] In addition, ASIC3 is expressed in various somatosensory neurons involved in nociception, proprioception, and mechanotransduction.[49,50] In DRG proprioceptors, ASIC3 is known as the molecular determinant involved in tether-mode mechanotransduction, by which mechanically sensitive ion channels are activated by neurite stretching via the extracellular matrix and cytoskeleton proteins tethering to the channels.[23] To our knowledge, no previous study has reported that ASIC3 was specifically involved in US neuromodulation. Notably, Kubanek et al found that pulsed US-activated MEC-4, a homolog of ASIC3, elicited robust reversal behavior in wild-type *Caenorhabditis elegans* nematodes in a pressure-, duration-, and pulse-protocol-dependent manner. The US responses were preserved in mutants unable to sense thermal fluctuations and absent in mutants (eg, *mec-4*) that lacked neurons required for mechanosensation.[44] Interestingly, a recent study showed that low-intensity ultrasound can modulate ASIC1a-dependent neural activity in mouse brains and cultured cortical neurons in vitro.[27] Based on this finding, we found that tUS-induced analgesia was partially affected in *ASIC1a*$^{-/-}$ mice in the ICS model, suggesting that heteromeric ASIC1a/ASIC3 channels may be involved in tUS analgesia in muscle afferents. Further studies should investigate how the tUS condition we applied can activate tether mechanotransduction in muscle so that the clinical applications of tUS can be further optimized and adjusted for different tissues in terms of the tissue type, size, and depth.

Although we have revealed that ASIC3 is the principal molecular determinant involved in tUS analgesia, it is intriguing to note that ASIC3 is also the molecular determinant involved in chronic pain in 3 mouse models of fibromyalgia induced by intramuscular acidification (the Sluka model), ICS, and repeated intermittent sound stress.[3,7,51] As mentioned above, ASIC3 is expressed in a wide range of DRG neurons, so it is rational to hypothesize that at least 2 ASIC3-positive neuron populations are involved in pronociceptive and antinociceptive functions. The ASIC3-containing channels in the pronociceptive and antinociceptive ASIC3 neurons may be composed of homomeric ASIC3 channels or heteromeric ASIC3 channels containing different ASIC subtypes. In the ICS model, knockouts of *ASIC1a*, *ASIC2b*, or *ASIC3* abolished tUS analgesia, suggesting that the heteromeric ASIC3 channels composed of ASIC1a, ASIC2b, and ASIC3 might be the substrate for tUS. In contrast, pronociceptive ASIC3-containing channels might be homomeric ASIC3 and/or heteromeric ASIC1b/ASIC3 channels. Nevertheless, further studies are required to confirm this hypothesis.

The role of NSAIDs in tUS analgesia is clinically important and requires further discussion. NSAIDs, such as diclofenac, are commonly used in pain relief patches and plasters and are combined with tUS in pain clinics. As shown in the Sluka model, aspirin, diclofenac, and ibuprofen partially reduced mechanical hyperalgesia. However, the combined use of aspirin and diclofenac would compromise tUS analgesia. In contrast, ibuprofen did not affect tUS. Voilley et al analyzed the effects of 13 NSAIDs on ASIC1a and ASIC3 activity and found that only flurbiprofen and ibuprofen selectively inhibited ASIC1a, and aspirin, salicylic acid, and diclofenac selectively inhibited ASIC3.[35] Although we only tested 3 NSAIDs in the Sluka model, the clinical use of aspirin, salicylic acid, and diclofenac is not recommended in tUS for different types of musculoskeletal pain.

This study had several limitations. First, the signaling pathways connecting substance P and ASIC3 should be elucidated in the future. Besides, we did not inject tracer in the treated GM, so we were not sure that the pERK expression neurons were actually muscle afferents. In addition, the analgesic mechanism of tUS in other tissues, such as tendons, joints, and fascia, deserves further research. Whether other mechano-stimulating modalities, such as shockwave, employ the same analgesic mechanism as tUS merits further study.

Authors' Contributions

> D-S H and **C-C C:** Designed the study, analyzed and interpreted the data, and drafted the work. **C-HL, Y-DS, K-VC, S-HL, Y-CC**, and **J-WW**: Performed the study and analyzed the data. All authors: Reviewed and approved the submitted version.

Availability of data and materials

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

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References

1. Clauw D: Fibromyalgia: A clinical review. JAMA Intern Med 311:1547-1555, 2014

2. Brum ES, Becker G, Fialho MFP, Oliveira SM: Animal models of fibromyalgia: What is the best choice? Pharmacol Ther 230:107959

3. Hsu W-H, Lee C-H, Chao Y-M, Kuo C-H, Ku W-C, Chen C-C, Lin Y-L: ASIC3-dependent metabolomics profiling of serum and urine in a mouse model of fibromyalgia. Sci Rep 9:12123, 2014

4. Sluka KA, Kalra A, Moore SA: Unilateral intramuscular injections of acidic saline produce a bilateral, long-lasting hyperalgesia. Muscle Nerve 24:37-46, 2014

5. Chang CT, Fong SW, Lee CH, Chuang YC, Lin SH, Chen CC: Involvement of acid-sensing ion channel 1b in the development of acid-induced chronic muscle pain. Fron Neurosci 13:1247, 2014

6. Chen W, Lee C, Lin S, Wong C, Sun W, Wood J, Chen C: Roles of ASIC3, TRPV1, and Nav1.8 in the transition from acute to chronic pain in a mouse model of fibromyalgia. Mol Pain 10:40, 2014

7. Sluka KA, Price MP, Breese NM, Stucky CL, Wemmie JA, Welsh MJ: Chronic hyperalgesia induced by repeated acid injections in muscle is abolished by the loss of ASIC3, but not ASIC1. Pain 106:229-239, 2014

8. Lin C-CJ, Chen W-N, Chen C-J, Lin Y-W, Zimmer A, Chen C-C: An antinociceptive role for substance P in acid-induced chronic muscle pain. Proc Natl Acad Sci 109:E76-E83, 2014

9. Chang CT, Jiang BY, Chen CC: Ion channels involved in substance P-mediated nociception and antinociception. Int J Mol Sci 20:1596, 2014

10. Vaught JL: Substance P antagonists and analgesia: A review of the hypothesis. Life Sci 43:1419-1431, 2014

11. Han D-S, Lee C-H, Shieh Y-D, Chen C-C: Involvement of substance P in the analgesic effect of low-level laser therapy in a mouse model of chronic widespread muscle pain. Pain Med 20:1963-1970, 2014

12. Coradini JiG, Mattjie TF, Bernardino GR, Peretti AL, Kakihata CMM, Errero TK, Escher AR, Bertolini GRF: Comparação entre o laser de baixa potência, ultrassom terapêutico e associação, na dor articular em ratos Wistar. Rev Bras Reumatol 54:07-12, 2014

13. ter Haar G: Therapeutic ultrasound. Eur J Ultrasound 9:3-9, 2014

14. Falconer J, Hayes KW, Chang RW: Effect of ultrasound on mobility in osteoarthritis of the knee: a randomized clinical trial. Arthritis Care Res 5:29-35, 2014

15. Özgönenel L, Aytekin E, Durmuşoğlu G: A double-blind trial of clinical effects of therapeutic ultrasound in knee osteoarthritis. Ultrasound Med Biol 35:44-49, 2014

16. Baker KG, Robertson VJ, Duck FA: A review of therapeutic ultrasound: Biophysical effects. Phys Ther 81:1351-1358, 2014

17. Yavuz F, Duman I, Taskaynatan MA, Tan AK: Low-level laser therapy versus ultrasound therapy in the treatment of subacromial impingement syndrome: A randomized clinical trial. J Back Musculoskelet Rehabil 27:315-320, 2014

18. Martins CC, Reis AS, da Motta KP, Luchese C, Wilhelm EA: Mechanistic pathways of fibromyalgia induced by intermittent cold stress in mice is sex-dependently. Brain Res Bull 187:11-23, 2014

19. Zimmer A, Zimmer A, Baffi J, Usdin T, Reynolds K, Konig M, Palkovits M, Mezey E: Hypoalgesia in mice with a targeted deletion of the tachykinin 1 gene. Proc Natl Acad Sci USA 95:2630-2635, 2014

20. De Felipe C, Herrero JF, O'Brien JA, Palmer JA, Doyle CA, Smith AJ, Laird JM, Belmonte C, Cervero F, Hunt SP: Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. Nature 392:394-397, 2014

21. Wu P-Y, Huang Y-Y, Chen C-C, Hsu T-T, Lin Y-C, Weng J-Y, Chien T-C, Cheng IH, Lien C-C: Acid-sensing ion channel-1a is not required for normal hippocampal LTP and spatial memory. J Neurosci 33:1828-1832, 2014

22. Lin S-H, Chien Y-C, Chiang W-W, Liu Y-Z, Lien C-C, Chen C-C: Genetic mapping of ASIC4 and contrasting phenotype to ASIC1a in modulating innate fear and anxiety. Eur J Neurosci 41:1553-1568, 2014

23. Lin S-H, Cheng Y-R, Banks RW, Min M-Y, Bewick GS, Chen C-C: Evidence for the involvement of ASIC3 in sensory mechanotransduction in proprioceptors. Nat Commun 7:11460, 2014

24. Nishiyori M, Ueda H: Prolonged gabapentin analgesia in an experimental mouse model of fibromyalgia. Mol Pain 42008. 52-52

25. Mazzuca M, Heurteaux C, Alloui A, Diochot S, Baron A, Voilley N, Blondeau N, Escoubas P, Gélot A, Cupo A, Zimmer A, Zimmer AM, Eschalier A, Lazdunski M: A tarantula peptide against pain via ASIC1a channels and opioid mechanisms. Nat Neurosci 10:943-945, 2014

26. Chu Y-C, Lim J, Chien A, Chen C-C, Wang J-L: Activation of mechanosensitive ion channels by ultrasound. Ultrasound Med Biol 48:1981-1994, 2014

27. Lim J, Tai H-H, Liao W-H, Chu Y-C, Hao C-M, Huang Y-C, Lee C-H, Lin S-S, Hsu S, Chien Y-C, Lai D-M, Chen W-S, Chen C-C, Wang J-L: ASIC1a is required for neuronal activation via low-intensity ultrasound stimulation in mouse brain. eLife 10:e61660

28. Julius D: TRP channels and pain. Annu Rev Cell Dev Biol 29:355-384, 2014

29. Cheng Y-R, Jiang B-Y, Chen C-C: Acid-sensing ion channels: Dual function proteins for chemo-sensing and mechano-sensing. J Biomed Sci 25:46, 2014

30. Chuang Y-C, Chen C-C: Force from filaments: The role of the cytoskeleton and extracellular matrix in the gating of mechanosensitive. Channels 102022.

31. Escoubas P, De Weille JR, Lecoq A, Diochot S, Waldmann R, Champigny G, Moinier D, Ménez A, Lazdunski M: Isolation of a tarantula toxin specific for a class of proton-gated Na+ channels. J Biol Chem 275:25116-25121, 2014

32. Diochot S, Baron A, Salinas M, Douguet D, Scarzello S, Dabert-Gay AS, Debayle D, Friend V, Alloui A, Lazdunski M, Lingueglia E: Black mamba venom peptides target acid-sensing ion channels to abolish pain. Nature 490:552-555, 2014

33. Diochot S, Baron A, Rash LD, Deval E, Escoubas P, Scarzello S, Salinas M, Lazdunski M: A new sea anemone

UNCORRECTED PROOF

peptide, APETx2, inhibits ASIC3, a major acid-sensitive channel in sensory neurons. EMBO J 23:1516-1525, 2014

34. Han DS, Lee CH, Shieh YD, Chang CT, Li MH, Chu YC, Wang JL, Chang KV, Lin SH, Chen CC: A role for substance P and acid-sensing ion channel 1a in prolotherapy with dextrose-mediated analgesia in a mouse model of chronic muscle pain. Pain 163:e622-e633, 2014

35. Voilley N, de Weille J, Mamet J, Lazdunski M: Nonsteroid anti-inflammatory drugs inhibit both the activity and the inflammation-induced expression of acid-sensing ion channels in nociceptors. J Neurosci 21:8026-8033, 2014

36. Chen W, Chen C: Acid mediates a prolonged antinociception via substance P signaling in acid-induced chronic widespread pain. Mol Pain 10:30, 2014

37. Ikeda H, Heinke B, Ruscheweyh R, Sandkuhler J: Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. Science 299:1237-1240, 2014

38. Babenko V, Graven-Nielsen T, Svensson P, Drewes A, Jensen T, Arendt-Nielsen L: Experimental human muscle pain induced by intramuscular injections of bradyknin, serotonin, and substance P. Eur J Pain 3:93-102, 2014

39. Linley J, Ooi L, Pettinger L, Kirton H, Boyle J, Peers C, Gamper N: Reactive oxygen species are second messengers of neurokinin signaling in peripheral sensory neurons. Proc Natl Acad Sci USA 109:E1578-E1586, 2014

40. Blackmore J, Shrivastava S, Sallet J, Butler CR, Cleveland RO: Ultrasound neuromodulation: A review of results, mechanisms and safety. Ultrasound Med Biol 45:1509-1536, 2014

41. Kamimura HAS, Conti A, Toschi N, Konofagou EE: Ultrasound neuromodulation: Mechanisms and the potential of multimodal stimulation for neuronal function assessment. Front Phys 8:150, 2014

42. Holland CK, Apfel RE: Thresholds for transient cavitation produced by pulsed ultrasound in a controlled nuclei environment. Journal Acoust Soc Am 88:2059-2069, 2014

43. Kubanek J, Shi J, Marsh J, Chen D, Deng C, Cui J: Ultrasound modulates ion channel currents. Sci Rep 6:24170, 2014

44. Kubanek J, Shukla P, Das A, Baccus SA, Goodman MB: Ultrasound elicits behavioral responses through mechanical effects on neurons and ion channels in a simple nervous system. Journal Neurosci Off J Soc Neurosci 38:3081-3091, 2014

45. Lim J, Chu Y-C, Hao C-M, Liao W-H, Lin S-S, Hsu S, Tai H-H, Chien Y-C, Lai D-M, Chen W-S, Chen C-C, Wang J-L: ASIC1a is required for neuronal activation via low-intensity ultrasound stimulation in mouse brain. bioRxiv 2020. 2020.2007.2010.196634

46. Yang Y, Pacia CP, Ye D, Zhu L, Baek H, Yue Y, Yuan J, Miller MJ, Cui J, Culver JP, Bruchas MR, Chen H: Sonothermogenetics for noninvasive and cell-type specific deep brain neuromodulation. Brain Stimul 14:790-800, 2014

47. Kellenberger S, Schild L: Epithelial sodium channel/degenerin family of ion channels: A variety of functions for a shared structure. Physiol Rev 82:735-767, 2014

48. Abdelhamid RE, Sluka KA: ASICs mediate pain and inflammation in musculoskeletal diseases. Physiology 30:449-459, 2014

49. Lin J-H, Hung C-H, Han D-S, Chen S-T, Lee C-H, Sun W-Z, Chen C-C: Sensing acidosis: Nociception or sngception? J Biomed Sci 25:85, 2014

50. Deval E, Lingueglia E: Acid-sensing ion channels and nociception in the peripheral and central nervous systems. Neuropharmacology 94:49-57, 2014

51. Hung C-H, Lee C-H, Tsai M-H, Chen C-H, Lin H-F, Hsu C-Y, Lai C-L, Chen C-C: Activation of acid-sensing ion channel 3 by lysophosphatidylcholine 16:0 mediates psychological stress-induced fibromyalgia-like pain. Ann Rheum Dis 79:1644-1656, 2014

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