

# Preventing the induction of acid saline-induced fibromyalgia pain in mice by electroacupuncture or APETx2 injection

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## Abstract

**Background:** Fibromyalgia (FM) is a syndrome involving chronic pain, fatigue, sleep difficulties, morning stiffness and muscle cramping lasting longer than 3 months. The epidemiological prevalence is approximately 3–5% in women and increases with age. Antagonism of acid-sensing ion channel 3 (ASIC3) reportedly attenuates acid saline-induced FM pain in mice.

**Aims:** Whether pre-treatment with electroacupuncture (EA) or APETx2 can attenuate mechanical hyperalgesia in this murine model remains unknown.

**Methods:** Accordingly, we examined the analgesic effect of EA in a murine model of FM pain induced by dual injections of acid saline and investigated whether EA or APETx2 can attenuate FM pain via the ASIC3 channel.

**Results:** EA significantly reduced mechanical hyperalgesia in this model. ASIC3 antagonism, induced by injecting APETx2, also significantly reduced mechanical hyperalgesia. The expression of ASIC3 in the dorsal root ganglia, spinal cord and thalamus was increased after FM model induction. Over-expression of these nociceptive channels was attenuated by pre-treatment with EA or an ASIC3 antagonist.

**Conclusion:** Our data reveal that both EA and ASIC3 blockade significantly reduce FM pain in mice via the ASIC3, Nav1.7 and Nav1.8 signalling pathways. Moreover, our findings support the potential clinical use of EA for the treatment of FM pain.

## Keywords

acid-sensing ion channel, dorsal root ganglion, electroacupuncture, fibromyalgia pain, sodium channel, spinal cord, thalamus

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## Introduction

The symptoms of fibromyalgia (FM) include widespread pain, fatigue, sleep difficulties, digestive disorders, headaches and burning sensations of the skin. FM predominantly occurs in women, at a ratio of 9:1 compared with men. The prevalence is 3–5% in women and 0.5% in men, and it increases with age.<sup>1,2</sup> Provisional criteria are a widespread pain index  $\geq 6$  and symptom severity score  $\geq 9$  for more than 3 months.<sup>2</sup> FM can be initiated by dual acid saline injections in mice with phenotypes including chronic widespread pain, fatigue, sleep disturbance and depression lasting longer than 2 weeks.<sup>3,4</sup> Vas et al. reported that acupuncture is an efficacious treatment for FM.<sup>5</sup>

The classes of proton-gated cation channels in the nervous system include the acid-sensing ion channels (ASICs) 1a, 1b, 2a, 2b, 3 and 4. Among these ion channels, ASIC3 is not only the most sensitive pH sensor in the ASIC family<sup>6</sup> but also detects mechanosensation and synaptic transmission. ASIC3 is largely expressed in the peripheral nervous system, with low levels of expression in the central nervous system (CNS). The ASIC3-specific antagonist APETx2 has an analgesic effect. Sluka et al.<sup>7</sup> reported that chronic hyperalgesia induced by repeated acid injections in muscle is abolished by the loss of ASIC3 but not of ASIC1. Chen et al.<sup>8</sup> indicated that the activation of ASIC3 is essential for the development of acid saline-induced FM in a murine model. Yen et al.<sup>9</sup> also revealed that ASIC3-mediated mechanisms are crucial in the treatment of FM-induced mechanical hyperalgesia. Painful stimuli often activate voltage-gated sodium channels to initiate action potentials in dorsal root ganglion (DRG) and spinal cord (SC) neurons. Nav1.7 and Nav1.8 are mainly involved in inflammatory and FM pain.<sup>9,10</sup> Yen et al.<sup>9</sup> suggested that overexpression of Nav1.7 and Nav1.8 can be reduced by ASIC3 gene deletion, suggesting a relationship between ASIC3, Nav1.7 and Nav1.8.

Acupuncture has been widely used for over 3000 years to alleviate many diseases. Recently, evidence-based studies have suggested that electroacupuncture (EA) can be used to treat stroke-induced dementia,<sup>11</sup> epilepsy,<sup>12</sup> changes in body weight<sup>13</sup> and pain.<sup>9,14,15</sup> Acupuncture significantly increases the release of endogenous opiates,<sup>16</sup> serotonin<sup>17</sup> and adenosine<sup>18</sup> to reduce pain by regulating several ion channels and receptors, including NMDA, ASIC3, TRPV1, TRPV4 and Nav channels.<sup>19–22</sup> In the current study, we aimed to examine the effect of electroacupuncture (EA) on the induction of FM pain in mice. We also tested whether the injection of the ASIC3 antagonist APETx2 at ST36 could relieve FM pain in mice.

## Methods

### Animals

All experiments were conducted on female C57/B6 mice (aged 8–12 weeks) purchased from BioLASCO Co, Ltd,

Taipei, Taiwan. The mice were randomly subdivided into four groups (n=8 per group): (1) healthy control (Normal group), (2) untreated FM model (FM group), (3) FM model receiving EA (FM+EA group), and (4) FM model receiving ASIC3 antagonist injection (FM+APETx2 group). Assuming an effect size of 0.6 in withdrawal threshold,  $\alpha$  of 0.05 and 80% power it was estimated that eight animals per group would be required. After arrival, mice were housed under a 12/12 hour light/dark cycle with ad libitum water and food. All procedures were approved by the Institute of Animal Care and Use Committee of China Medical University (permit no. 2016-061) and conducted in accordance with the Guide for the Use of Laboratory Animals of the National Research Council and the ethical guidelines of the International Association for the Study of Pain. The number of animals used and their suffering were minimised. The laboratory workers were kept blind to treatment allocation during the experiments and analysis.

### Fibromyalgia pain model and pharmacological injection

The mice were anaesthetised with 1% isoflurane and received dual injections of 20  $\mu$ L acid saline into the right gastrocnemius muscle on day 0 and day 5 (pH 4, buffered with 20 mM HEPES) to model FM pain. Behavioural tests were conducted 14 days after induction of FM pain. A dose of 20 pmol APETx2 (in 20  $\mu$ L acid saline with a concentration of 1  $\mu$ M APETx2) was injected 15 min before the second acid saline injection at ST36 under light isoflurane anaesthesia (1%).

### Electroacupuncture treatment

Animals were anaesthetised with 2% isoflurane for EA treatment. EA was applied 15 min before the second acid saline injection using stainless steel needles (0.5 inch, 32 G, Yu Kuang, Taiwan) inserted into the muscle layer to a depth of 2–3 mm at bilateral ST36. ST36 is located on the tibialis anterior muscle, approximately 1/6th of the distance from the patella to the lateral malleolus. A stimulator (Trio 300, Ito, Japan) delivered 100  $\mu$ s square pulses of 1 mA for 15 min at 2 Hz.

### Mechanical hyperalgesia

Mechanical hyperalgesia was tested 14 days after the first intramuscular injection of acid saline. All the experiments were performed at room temperature (approximately 25°C) and the stimuli were applied only when the animals were calm but not sleeping or grooming. Mechanical sensitivity was measured by testing the force of responses to stimulation with three applications of electronic von Frey filaments (North Coast Medical, Gilroy, CA, USA).

### Immunoblotting assay

Animals were anaesthetised with 2% isoflurane followed by cervical dislocation. Dorsal root ganglia (DRG), spinal cord (SC) and thalamus were immediately excised to extract proteins on day 14 after the first acid saline injection. Total proteins were prepared by homogenising tissue in lysis buffer containing 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 0.02%  $\text{NaN}_3$  and 1× protease inhibitor cocktail (AMRESCO). The extracted proteins (30  $\mu\text{g}$  per sample assessed by BCA protein assay) were subjected to 8% SDS-Tris glycine gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk in TBS-T buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20), incubated with primary antibody (Alomone Labs Ltd) in TBS-T with 1% bovine serum albumin, and incubated for 1 hour at room temperature. Peroxidase-conjugated anti-rabbit antibody (1:5000) was used as a secondary antibody. The bands were visualised by an enhanced chemiluminescent substrate kit (Pierce) with LAS-3000 Fujifilm (Fuji Photo Film Co Ltd). Where applicable, the image intensities of specific bands were quantified with National Institutes of Health (NIH) ImageJ software (Bethesda, MD, USA).

### Statistical analysis

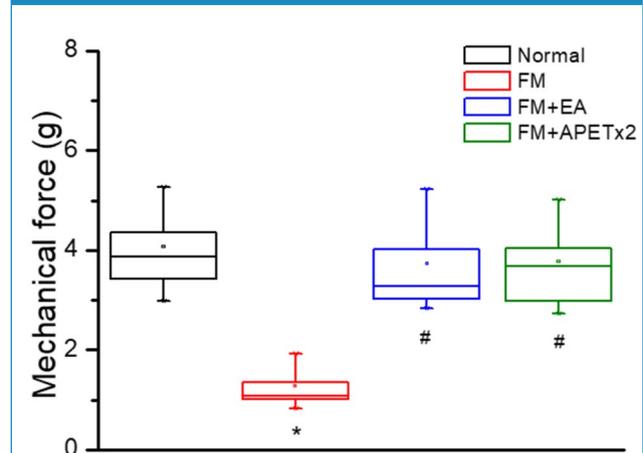
All statistical data are presented as mean  $\pm$  SE. Statistically significant differences between the Normal, FM, FM+EA and FM+APETx2 groups were examined for using analysis of variance (ANOVA), followed by post hoc Tukey's tests ( $P < 0.05$  was considered statistically significant).

### Results

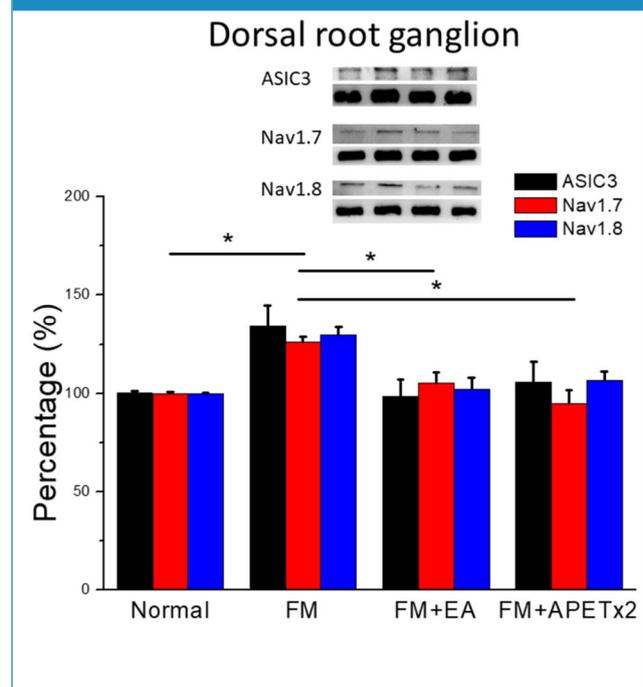
To examine the effect of EA and APETx2—an ASIC3 antagonist—on the acid saline-induced FM murine model, we injected acid saline into the gastrocnemius muscle. In control mice, normal saline did not induce mechanical hyperalgesia on day 14 (Figure 1, mechanical force =  $4.07 \pm 0.25$  g,  $n=8$ ). On day 14 after FM induction, mechanical hyperalgesia was significantly induced (Figure 1,  $1.27 \pm 0.12$  g,  $n=8$ ). Next, we demonstrated that 2 Hz EA reduced the acidic saline-induced mechanical hyperalgesia on day 14 (Figure 1,  $3.74 \pm 0.30$  g,  $n=8$ ). EA may reduce FM pain through the ASIC3 pathway. Our results indicate that administration of the ASIC3 antagonist APETx2 significantly reduced FM pain in mice (Figure 1,  $3.77 \pm 0.30$  g,  $n=8$ ).

To identify whether EA or APETx2 could prevent the overexpression of ASIC3 and Navs in the DRG of mice, we performed Western blot analyses. ASIC3 was expressed in normal DRG (Figure 2,  $100.11 \pm 0.97\%$ ,  $n=6$ ) and the level of

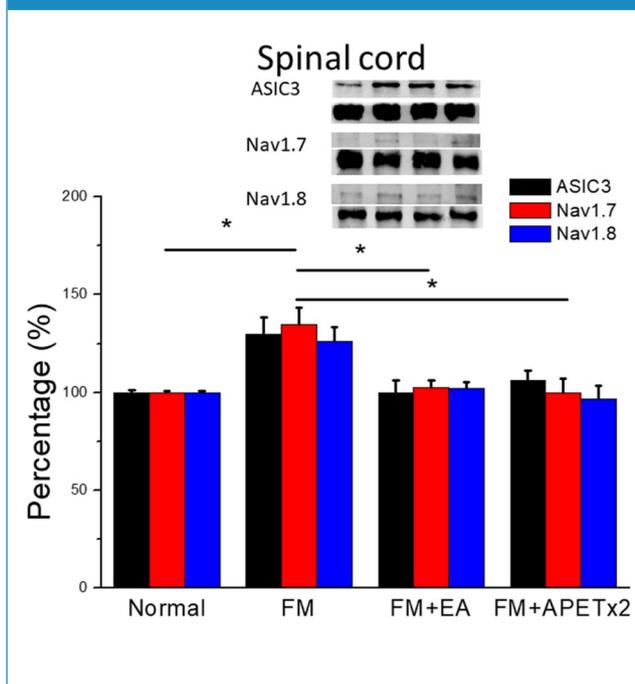
**Figure 1.** Mechanical force in the hind-paws of mice receiving normal saline injection (Normal group,  $n=8$ ) or acid saline injections and remaining untreated (FM group,  $n=8$ ) or receiving 2 Hz EA (FM+EA group,  $n=8$ ) or APETx2 (ASIC3 antagonist) injection (FM+APETx2 group,  $n=8$ ). \* $P < 0.05$  versus Normal group. #  $P < 0.05$  versus FM group. EA, electroacupuncture. FM, fibromyalgia.



**Figure 2.** Expression levels of ASIC3, Nav1.7 and Nav1.8 in the dorsal root ganglion of mice from the Normal (control), FM, FM+EA and FM+APETx2 groups (from left to right). \* $P < 0.05$  between groups. The Western blot bands at the top show the target protein. The lower bands are internal controls ( $\alpha$ -tubulin). APETx2 = ASIC3 antagonist. EA, electroacupuncture. FM, fibromyalgia.



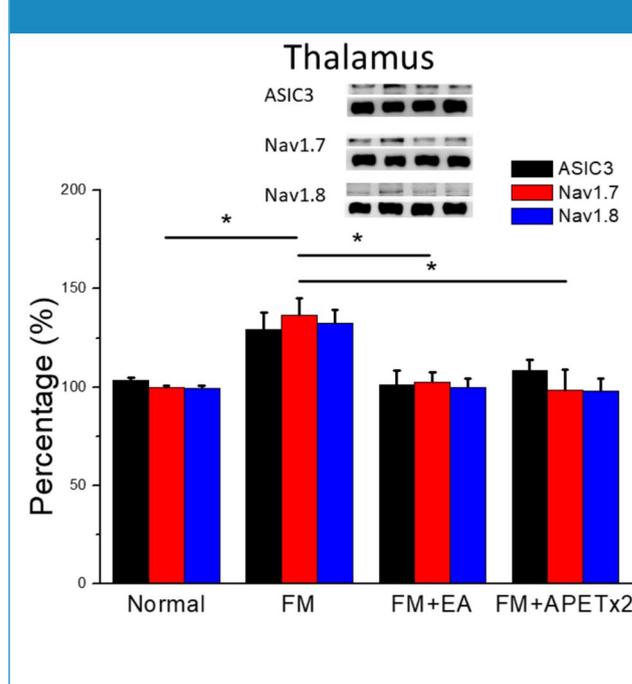
**Figure 3.** Expression levels of ASIC3, Nav1.7 and Nav1.8 in the spinal cord of mice from the Normal (control), FM, FM+EA and FM+APETx2 groups (from left to right). \* $P < 0.05$  between groups. The Western blot bands at the top show the target protein. The lower bands are internal controls ( $\alpha$ -tubulin). APETx2 = ASIC3 antagonist. EA, electroacupuncture. FM, fibromyalgia.



expression increased following acid saline injection (Figure 2,  $134.2 \pm 10.36\%$ ,  $n=6$ ). EA at 2 Hz significantly reduced ASIC3 overexpression (Figure 2,  $98.65 \pm 8.65\%$ ,  $n=6$ ) and levels similar to the normal group were observed in the DRG of the FM+APETx2 group (Figure 2,  $105.65 \pm 10.36\%$ ,  $n=6$ ). We also assessed the effects of EA on sodium channels. Western blot analyses demonstrated increased levels of Nav1.7 in the FM group (Figure 2,  $126.36 \pm 2.69\%$ ,  $n=6$ ). Nav1.7 levels were attenuated in the FM+EA (Figure 2,  $105.36 \pm 5.36\%$ ,  $n=6$ ) and FM+APETx2 (Figure 2,  $94.68 \pm 6.86\%$ ,  $n=6$ ) groups. A similar effect was observed for Nav1.8 (Figure 2, FM:  $129.63 \pm 3.98\%$ , EA:  $102.36 \pm 5.68\%$ , APETx2:  $106.65 \pm 4.65\%$ ,  $n=6$ ). These data indicate the crucial role of ASIC3 in this murine model of FM and indicate that EA may regulate the ASIC3 pathway.

To determine whether EA can downregulate ASIC3 or Navs in the CNS, we examined the effect of injecting acid saline on the expression levels of ASIC3 or Navs in the SC and thalamus. ASIC3 was expressed in the normal SC (Figure 3,  $100.21 \pm 1.03\%$ ,  $n=6$ ) and the level of expression increased following the injection of acid saline (Figure 3,  $129.65 \pm 8.65\%$ ,  $n=6$ ). EA attenuated the overexpression of ASIC3 in the SC (Figure 3,  $99.65 \pm 6.38\%$ ,  $n=6$ ). Results were similar in the FM+APETx2 treated mice (Figure 3,  $106.32 \pm 4.65\%$ ,  $n=6$ ). Moreover, Nav1.7 expression, which

**Figure 4.** Expression levels of ASIC3, Nav1.7 and Nav1.8 in the thalamus of mice from the Normal (control), FM, FM+EA and FM+APETx2 groups (from left to right). \* $P < 0.05$  between groups. The Western blot bands at the top show the target protein. The lower bands are internal controls ( $\alpha$ -tubulin). APETx2 = ASIC3 antagonist. EA, electroacupuncture. FM, fibromyalgia.



was increased in the FM mice (Figure 3,  $134.65 \pm 8.91\%$ ,  $n=6$ ), was reduced by treatment with EA (Figure 3,  $102.63 \pm 3.82\%$ ,  $n=6$ ) or APETx2 (Figure 3,  $99.65 \pm 7.31\%$ ,  $n=6$ ). A similar pattern was found for Nav1.8 (Figure 3, FM:  $126.32 \pm 7.2\%$ , EA:  $102.36 \pm 2.9\%$ , APETx2:  $96.68 \pm 6.9\%$ ,  $n=6$ ). ASIC3 expression, which increased in the thalamus of FM mice (Figure 4, ASIC3:  $129.46 \pm 8.63\%$ ,  $n=6$ ), was attenuated by treatment with EA or APETx2 (Figure 4, FM+EA:  $101.36 \pm 6.89\%$ , FM+APETx2:  $108.36 \pm 5.68\%$ ,  $n=6$ ). These results indicate that the ASIC3 signalling pathway is crucial in the CNS of mice with FM pain. A similar pattern was found in the thalamus for Nav1.7 (Figure 4, FM:  $136.65 \pm 8.36\%$ , FM+EA:  $102.69 \pm 4.68\%$ , FM+APETx2:  $98.63 \pm 10.23\%$ ,  $n=6$ ) and Nav1.8 (Figure 4, FM:  $132.36 \pm 6.98\%$ , FM+EA:  $99.65 \pm 4.69\%$ , FM+APETx2:  $97.86 \pm 6.35\%$ ,  $n=6$ ).

## Discussion

Accumulating evidence suggests that the mechanical hyperalgesic priming of nociceptors, especially ASIC3 and Navs, is crucial for the transition of chronic pain.<sup>8,9</sup> In inflammatory pain, nociceptive priming depends on intracellular signalling pathways from protein kinase A (PKA) to protein kinase C epsilon type (PKC $\epsilon$ ).<sup>23,24</sup> ASIC3 and

Navs are the major ion channels in the acid-induced murine fibromyalgia model. Chen et al.<sup>20</sup> demonstrated the inhibitory effects of APETx2 on FM pain and Nav currents. Our results indicate that EA significantly attenuates FM pain. EA also reduced ASIC3 and Navs in tissue from the peripheral DRG to the central SC and thalamus.

Recent reports indicate that EA reduces pain by increasing the release of endogenous opiates,<sup>16</sup> serotonin<sup>17</sup> and adenosine.<sup>18</sup> Han reported that EA increases the release of endogenous morphine, enkephalin and dynorphin over a range of frequencies.<sup>16</sup> Hamza et al. also suggested that transcutaneous electrical acupuncture point stimulation (TEAS) reduces opioid intake and opioid-related side effects following surgery.<sup>25</sup> EA pre-treatment alleviates postoperative analgesic requirements and side effects in patients undergoing lower abdominal surgery.<sup>26</sup> Acupuncture increases the release of local adenosine for relief from inflammatory and neuropathic pain.<sup>17</sup> Yen et al.<sup>9</sup> revealed that EA significantly reduces mechanical hyperalgesia by reducing ASIC3 during the sub-acute phase of FM pain in a murine model. The effect of EA was abolished by opioid and adenosine receptor antagonists. The results obtained by the administration of opioid and adenosine receptor antagonists and those obtained by EA are similar.<sup>9</sup> Herein we indicated that ASIC3, Nav1.7 and Nav1.8 are upregulated in the DRG, SC and thalamus by acid saline treatment and are recovered by EA and APETx2. We suggest that the mechanisms underlying this phenomenon are similar to those in a previous study showing that EA analgesia may operate via the ASIC3 pathway.<sup>9</sup>

The therapeutic effect of opioids for FM pain is still debatable. There is increasing evidence that opioids offer little help to FM patients suffering from widespread pain. In contrast, opioid administration can relieve the painful sensation in some patients. Furthermore,  $\mu$ - or  $\delta$ -opioid receptor agonists can significantly reduce mechanical pain in FM animal models,<sup>27</sup> as can glutamate receptor antagonists delivered to the SC.<sup>28</sup> The administration of neurotrophin-3 (NT-3) reduced acid saline-induced FM pain,<sup>29</sup> with results similar to those obtained using the calcium channel antagonist pregabalin and the M-type voltage-gated potassium channel activator flupirtine.<sup>30,31</sup> EA was reported to increase the expression of (NT-3) in the DRG and SC.<sup>32,33</sup> Acid saline-induced FM pain in mice is also associated with changes in the central thalamus.<sup>34</sup> Yen et al.<sup>9</sup> also reported that EA can reduce ASIC3 and Navs overexpression in the thalamus. Here, we suggest that pre-treatment with EA can also reduce the overexpression of ASIC3 and Navs.

In this study, it appeared that EA alleviated FM pain in mice by downregulating increased signalling of the ASIC3–Navs pathway between the peripheral DRG and the central SC and thalamus. Similar results were observed in the APETx2-injected group. These results

support the clinical application of EA or pharmacological injections of ASIC3 antagonists to reduce FM pain.

### Contributors

YWL and HCH conceived and designed the study. HCH and LTY employed the experiments, and collected and analysed the data. CLH and YWL wrote the manuscript. YWL obtained the research grants for the current study. All the authors agreed for submission and approved the final version of the manuscript accepted for publication.

### Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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