



Acupuncture modulates extracellular ATP levels in peripheral sensory nervous system during analgesia of ankle arthritis in rats

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Abstract

As an ancient analgesia therapy, acupuncture has been practiced worldwide nowadays. A good understanding of its mechanisms will offer a promise for its rational and wider application. As the first station of pain sensation, peripheral sensory ganglia express pain-related P2X receptors that are involved in the acupuncture analgesia mechanisms transduction pathway. While the role of their endogenous ligand, extracellular ATP (eATP), remains less studied. This work attempted to clarify whether acupuncture modulated eATP levels in the peripheral sensory nerve system during its analgesia process. Male Sprague–Dawley rats underwent acute inflammatory pain by injecting Complete Freund's Adjuvant in the unilateral ankle joint for 2 days. A twenty-minute acupuncture was applied to ipsilateral Zusanli acupoint. Thermal hyperalgesia and tactile allodynia were assessed on bilateral hind paws to evaluate the analgesic effect. eATP of bilateral isolated lumbar 4-5 dorsal root ganglia (DRGs) and sciatic nerves were determined by luminescence assay. Nucleotidases NTPDase-2 and -3 in bilateral ganglia and sciatic nerves were measured by real-time PCR to explore eATP hydrolysis process. Our results revealed that acute inflammation induced bilateral thermal hyperalgesia and ipsilateral tactile allodynia, which were accompanied by increased eATP levels and higher mechanosensitivity of bilateral DRGs and decreased eATP levels of bilateral sciatic nerves. Acupuncture exerted antinociception on bilateral hind paws, reversed the increased eATP and mechanosensitivity of bilateral DRGs, and restored the decreased eATP of bilateral sciatic nerves. NTPDase-2 and -3 in bilateral ganglia and sciatic nerves were inconsistently modulated during this period. These observations indicate that eATP metabolism of peripheral sensory nerve system was simultaneously regulated during acupuncture analgesia, which might open a new frontier for acupuncture research.

Keywords Acupuncture analgesia · Dorsal root ganglia · Sciatic nerves · Extracellular ATP · Nucleotidases

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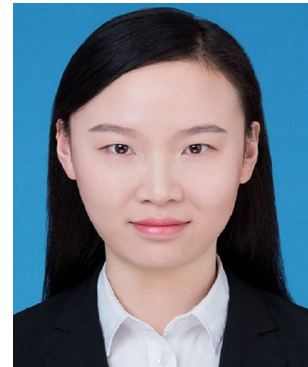
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Dan Shen graduated with a Ph.D. in Medicine from Shanghai University of Traditional Chinese Medicine. She is currently working at the School of Traditional Chinese Medicine, Naval Medical University. She is interested in exploring and confirming the efficacy of alternative and complementary therapies in the treatment of acute and chronic pain. She focuses on the role of purinergic signaling in the mechanisms of acupuncture analgesia. Her aim is to continue research in this area and provide reliable evidence for the role of purine signaling in pain neuromodulation.



Introduction

As an ancient approach for pain management, acupuncture (AP) is nowadays practiced worldwide in clinics. During AP, fine needles are inserted into some specific points (acupoints) and are manipulated to trigger biological signals. According to the WHO notes, AP has efficacy on pain superior to other diseases, including chronic and acute pain and peripheral and visceral pain [1]. Although to date, the analgesic effect of AP has been well documented in clinic; its biological basis is still not fully understood. A good understanding of its mechanisms will offer promise for its rational and wider application. Nerve system is the main target to be activated by AP during the anti-nociceptive process, from afferent nerve endings to cortex [2]. As the first station to receive nociceptive inputs, neurons in the peripheral sensory ganglia are the foremost target to be modulated by AP [3, 4].

The ATP neurotransmitter system is recognized as an important pathway in the periphery to the spinal cord. Extracellular ATP (eATP) and its hydrolyzed products play conflicting roles in pain transmission by binding to their corresponding purinoceptors. In general, solid evidence has shown that eATP is the endogenous ligand to P2X₃ (P2X_{2/3}), P2X₄, and P2X₇ receptors, which contribute to pain transmission [4, 5], and its hydrolyzed product ADP is the agonist of P2Y₁, P2Y₁₂, and P2Y₁₃ receptors, which boost painful sensation as well [4, 5]. While its down-stream product, adenosine (ADO), accounts for anti-nociception by binding to A₁ or A₃ receptors [6].

In peripheral sensory ganglia, all multiple P2X and P2Y receptors are expressed in neurons or glial cells, especially the P2X₃ receptors that are concentrated in pain-related small-diameter sensory neurons [7]. Besides, as the peripheral fibers of lumbar ganglia neurons, sciatic nerves express P2Y_{1, 6, 11} subtypes [8]. Among them, expression of P2X₃ (P2X_{2/3}), P2X₇, and P2Y_{1, 2, 6} receptors in ganglia is increased during peripheral neuropathic pain, inflammatory pain, or visceral pain [3, 4], especially P2X₃ receptors. Since Burnstock hypothesized in 2009 that beneficial effects of AP involved triggering the purinergic signaling cascade via ATP release in acupoints [9], researches on purinergic signaling were intensified in the field of AP analgesia. Tang et al. have reviewed that P2

purinoceptors, especially P2X₃ in peripheral ganglia, are the focus of these studies, and AP or EAP plays analgesic effect by reversing the elevated P2X₃ expression in different types of pain [3, 4].

Besides P2 purinoceptors, their ligands are another vital component of purinergic signaling. While the role of them remains less studied. In order to address this issue, we used the acute paw inflammatory model to investigate whether eATP levels of DRGs or sciatic nerves were modulated in the process of AP-induced analgesia. This study accelerates our understanding of the role of purinergic signaling in AP analgesia mechanism, which will open a new frontier for AP research.

Methods

Animals

Adult male SD rats weighted 200 ± 20 g were used. All rats were randomly grouped into control, model, and AP treated groups. For the behavioral tests, each group comprised of 6 rats. For the other experiments, each group comprised 3–12 rats. The rats were housed in a temperature-controlled (22 °C–24 °C) room with a 12/12 h light-dark cycle and ad libitum access to food and water. Before the tests, rats were allowed to acclimate to the housing facilities daily for 3 days. All experimental protocols were approved by the Animal Care Committee of Shanghai University of Traditional Chinese Medicine (Shanghai, China) (No: SZY201807008).

Acute adjuvant arthritis model

Peripheral inflammation pain was established by injection of 50 μ l Complete Freund's Adjuvant (CFA) into the left ankle joint cavity to induce acute adjuvant arthritis after rat was anesthetized with 1.5% isoflurane. Inflammatory syndrome, local swelling, and behave disability appeared within 24 h.

Behavioral tests

Pain thresholds of the bilateral hind plantar were determined to reflect the pain levels. Thermal hyperalgesia was assessed using radiant heat (IITC336G, IITC Life Science, USA), and the paw withdrawal latency (PWL) was defined as the time taken by the rat to remove its hind paw from the heat source. A cut off time of 20 s was applied to prevent potential tissue damage. Mechanical allodynia was evaluated by an electronic Von Frey anesthesiometer (IITC2450, IITC Life Science, USA), and the paw withdrawal threshold (PWT) was defined as the force to push the rat to withdraw its paw. Each heat or force stimulus was applied 3 times at 10 min intervals at each time point. Thermal hyperalgesia was measured 2 h after the mechanical allodynia assessment. The timeline for pain threshold measurement is illustrated in Fig. 1.

AP treatment

Unilateral AP treatment was performed on an affected side of conscious and unrestrained rats as described in our previous work [10]. The equivalent of the human acupoint Zusanli (ST36) was used in this study. ST36 is located at the posterolateral of knee joint, about 5 mm below fibula capitulum. Stainless steel needle (0.18 mm × 13 mm, Cloud Dragon Medical Equipment Company, China) was gently inserted at an approximate depth of 7 mm. To avoid variables as much as possible, all AP treatments were exerted by one person.

The AP procedure consisted of 30 s lifting-thrusting and 30 s twisting and lasted for 20 min, which was initiated on the 2nd day after CFA injection and repeated on the 3rd day (Fig. 1).

Tissue preparation

After the behavioral tests for 2nd AP treatment, rats were euthanasia executed with CO₂, and tissues were immediately isolated. In ganglia dissection, bilateral 4–5 lumbar (L4–5) DRGs in each group were dissected from the vertebral column according to the method described in Burkey et al. work [11]. In sciatic nerve dissection, in the middle part of the dorsal thigh of the hind limb, the skin was incised with a scalpel

along the sciatic nerve. After blunt separation of muscle, bilateral sciatic nerves were exposed and excised for 1 cm in length.

For eATP measurement, isolated tissues were equilibrated in artificial physiological solution (PS) for 2 h for RT-PCR; tissues were incubated in Trizol solution (Thermo Fisher, USA) and stored in liquid nitrogen.

Reagents and solutions

PS was used to bathe the excised tissues, which contained (in mM) 137 NaCl, 2.7 KCl, 2 CaCl₂, 5 MgCl₂, 5.6 glucose, and 10 HEPES, pH 7.4 (adjusted with NaOH). 6-N,N-Diethyl-β-γ-dibromomethylene-D-adenosine-5'-triphosphate trisodium salt hydrate (ARL67156) and luciferin-luciferase (L-L) assay mixture were procured from Sigma-Aldrich (USA). ARL67156 was prepared in stock solution with distilled water and stored at –20 °C and diluted into 100 μM working solution when used.

External stimuli

Excised ganglia were irritated by mechanical or chemical stimulus to determine their sensitivity to release ATP. Air bubbles were introduced to DRGs bathed in 100 μl PS. The bubbles were made by an electric pipette gun (Model: LH3 09 06, Thermo Fisher, USA), which blew 50 μl air per time at a rate of 14.9 μl/s and repeated 20 cycles. One hundred millimolar KCl in PS was used as chemical stimulus as described in the literature [12]. After irritation, 50 μl superfusate was aliquoted to assess ATP content.

ATP measurement

eATP levels were quantified by an L-L assay and expressed in molar concentration. To assess DRGs eATP, equilibrated L4–5 ganglia were transferred to 96 well-plate containing 100 μl PS/well. L-L assay mixture was diluted with dilution buffer and was adjusted to isotonicity with mannitol. One hundred microliter L-L was added to each well. To study the breakdown of exogenous ATP (exo-ATP) by sciatic nerves, nerves were transferred to 24 well-plate containing 2 μM exo-ATP in

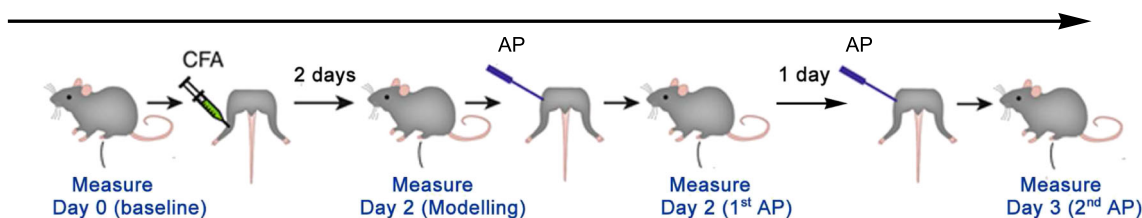


Fig. 1 Schematic diagram outlining the experimental process behavioral measurements and AP treatments in the acute inflammatory pain model. CFA administration in the left ankle joint was applied to induced acute inflammation on day 0. AP was given to the left ST36 2 days later and

repeated the next day. Pain thresholds were measured just before CFA injection (baseline), before 1st treatment (modeling), and after each treatment

400 μ l PS/well and incubated for 2 min. Subsequently, 50 μ l supernatant was aliquoted and mixed with 50 μ l L-L assay mix. The light emission was measured immediately by luminometer (Synergy Mx, BioTek, Winooski, USA).

In each experiment, calibration of luminescence *versus* ATP standards was performed with PS or PS containing corresponding compounds, which aimed to eliminate the interference of the reagents with L-L assay.

Quantitative real time-PCR

Quantitative real-time PCR (RT-PCR) was performed as described in our previous work [13]. Total mRNA was isolated from the L4-L5 DRGs or sciatic nerves. The primer for NTPDase-2 is sense strand, 5'-GGCCAAAGGGCTACTCTACC-3'; antisense strand, 5'-GTTCTGACAGGCTGACGAT-3'. The primer for NTPDase-3 is sense strand, 5'-ACGGTTACAGCACCACTTC-3'; antisense strand, 5'-ACAGCTGTGGGTACCAGTT-3'. The relative abundance of specific mRNA was determined by the $2^{-\Delta\text{Ct}}$ method, using β -actin as the internal standard.

Statistical analysis

The data were analyzed and expressed as mean \pm se. The figures were prepared by the Graphpad Prism 8.0 software (San Diego, California, USA). Statistical significance was carried out using SPSS 24.0 (Chicago, Illinois, USA). Differences among multiple groups were tested with one-way ANOVA, followed by Fisher's least significant difference (LSD). Where only two groups were compared, two sample *t*-test was used. $P < 0.05$ was considered statistically significant.

Results

Unilateral AP treatment relieved CFA-induced pain hypersensitivity of bilateral hind paws

Figure 2a, b showed the pain thresholds of ipsilateral hind paws. The baseline PWL and PWT (day 0) were similar among each group, 8.65 s–11.98 s for PWL and 40.47 g–55.60 g for PWT. Unilateral CFA-treated rats exhibited thermal hyperalgesia and tactile allodynia at least within 3 days ($n = 6$, CFA *vs.* control $P < 0.001$ for PWL and $P < 0.001$ for PWT). First AP treatment in ST36 significantly reversed PWL and PWT ($n = 6$, AP *vs.* CFA $P < 0.001$ for PWL and $P < 0.001$ for PWT). Repeated treatment in next day led to no further improvement.

To contralateral hind paws (Fig. 2c, d), thermal hyperalgesia rather than tactile allodynia was induced by ipsilateral injection of CFA ($n = 6$, CFA *vs.* control $P < 0.001$ for

PWL) and was also relieved by 1st AP treatment ($n = 6$, AP *vs.* CFA, $P < 0.001$ for PWL). Similarly, 2nd treatment had no further improvement.

Figure 3 compared the changes in the bilateral pain thresholds. Figure 3a2 revealed that CFA-induced thermal hyperalgesia in ipsilateral was transiently more severe than that in contralateral ($n = 6$, $P < 0.01$, on day 2 before 1st AP treatment), which continued until after 1st AP (Fig. 3a3). As described above, because of no tactile allodynia in contralateral paws, left hind paws showed constantly lower pain threshold than right paws ($n = 6$, $P < 0.001$, from day 2 to day 3) (Fig. 3b2). Although there was a significant difference in thermal hyperalgesia between bilateral hind paws after 1st AP (Fig. 3a3), further analysis showed that the AP-induced improvements in bilateral thermal hyperalgesia, together with contralateral tactile allodynia, had no significant difference (Fig. 3c), implying that AP had no preference on these hypersensitivities.

Unilateral AP treatment modulated eATP levels of bilateral inflammation-injured DRGs and sciatic nerves

ATP can be released from soma and terminal endings of DRG neurons [14, 15]. Hence, we wondered whether eATP of sensory neurons changed with the status of pain. At the end of the behavioral tests after 2nd AP, bilateral L4-5 DRGs and the main trunk of sciatic nerves were excised from each group to assess eATP levels.

For the ipsilateral DRGs, in a non-stimulated situation (Fig. 4a), eATP was 177.5 ± 6.3 pM in the control group, which significantly increased to 380 ± 26.8 pM in the CFA group ($n = 5$, $P < 0.001$ *vs.* control) and decreased to 266.7 ± 8.8 pM in the AP-treated group ($n = 3$, $P < 0.001$ *vs.* CFA). In order to identify whether injured DRGs become more sensitive to external stimuli, mechanical and chemical stimuli were delivered to L4-5 DRGs in each group. The results showed that stimulation by air bubbles led to excessive eATP in all groups (Fig. 4b), 685.6 ± 50.3 pM in control, 1814.0 ± 182.5 pM in the CFA group, and 840.8 ± 62.2 pM in the AP-treated group (Fig. 4d). As reported, 100 mM KCl could induce sciatic nerve-ligation-injured DRGs to elevate eATP [12]. In this work, the differences in eATP levels among each group remained in the presence of 100 mM KCl (Fig. 4c). However, further within-group comparisons demonstrated that mechanical stimulus rather than KCl had significant enhancement effects ($n = 6$ –12, $P < 0.01$ *vs.* non-stimulated group, $P < 0.01$ *vs.* chemical stimulus) (Fig. 4d).

The responses of contralateral DRGs were similar. eATP level in the CFA group was higher than that of the control and AP group in the absence of any stimuli (Fig. 5a). Mechanical stimulus rather than chemical one significantly elevated eATP

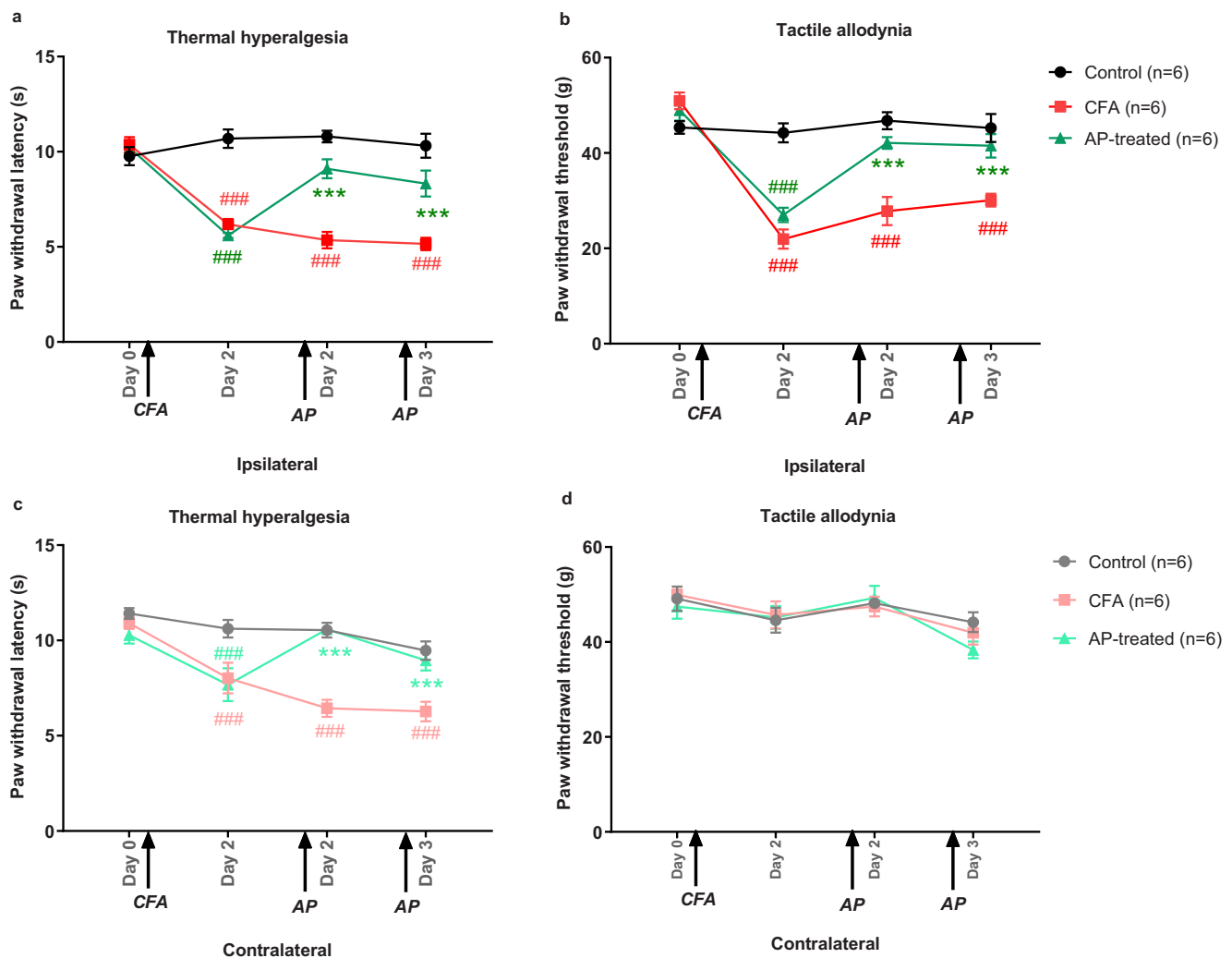


Fig. 2 The effects of AP treatment on CFA-induced bilateral pain sensation. **a, b** The changes of thermal and tactile sensation of ipsilateral hind paws, respectively. **c, d** The changes of thermal and tactile sensation of contralateral hind paws, respectively. Acute ankle arthritis was

established by injecting CFA in left hind ankle on day 0. AP treatment started on day 2 and was repeated on day 3 (see Fig. 1). One-way ANOVA followed by LSD was used. ### $P < 0.001$ vs. control group. *** $P < 0.001$ vs. CFA group

levels in each group ($n = 6–11$, $P < 0.001$ vs. non-stimulated group, $P < 0.001$ vs. chemical stimulus) (Fig. 5b–d).

Figure 6 showed the comparisons of eATP responses of bilateral DRGs to external stimuli. Regardless if the stimulus existed or not, the difference only occurred at CFA states. eATP levels in ipsilateral DRGs were always higher than that in contralateral ones ($n = 3–12$, $P < 0.001$). Hence, AP intervention had more beneficial influence on ipsilateral DRGs to reverse excessive eATP than contralateral ones and made corresponding adjustment according to the amplitude of ATP rise.

Sciatic nerve is the peripheral fiber trunk of DRG neurons. In the present work, one of its branches (tibial nerve) senses the inflammatory ankle; another branch (peroneal nerve) senses the ST36 acupoint. We found that bilateral sciatic nerves had a strong ability to hydrolyze exogenous ATP (exo-ATP) (Fig. 7). Sciatic nerves were bathed in 200 μ l

and 2 μ M exo-ATP for 2 min. Regarding ipsilateral sciatic nerves, around half (51%) of the exo-ATP was hydrolyzed by the control nerves. This ability was further strengthened in the CFA group (74%) ($n = 9–10$, $P < 0.001$ vs. control) and restored in the AP-treated group (57%) ($n = 10$, $P < 0.001$ vs. CFA group). The same phenomenon also was observed in contralateral nerves.

Unilateral AP modulated NTPDases mRNA expression in bilateral DRGs and sciatic nerves

Besides physiological release, hydrolysis is another vital way to control eATP levels. Our previous work indicated that rat DRGs expressed ecto-ATPase that hydrolyzed eATP released from DRGs themselves [16] or the co-cultured mast cells [13]. The present work corroborated such finding again. As we described in Fig. 4b, mechanical stimulus induced higher

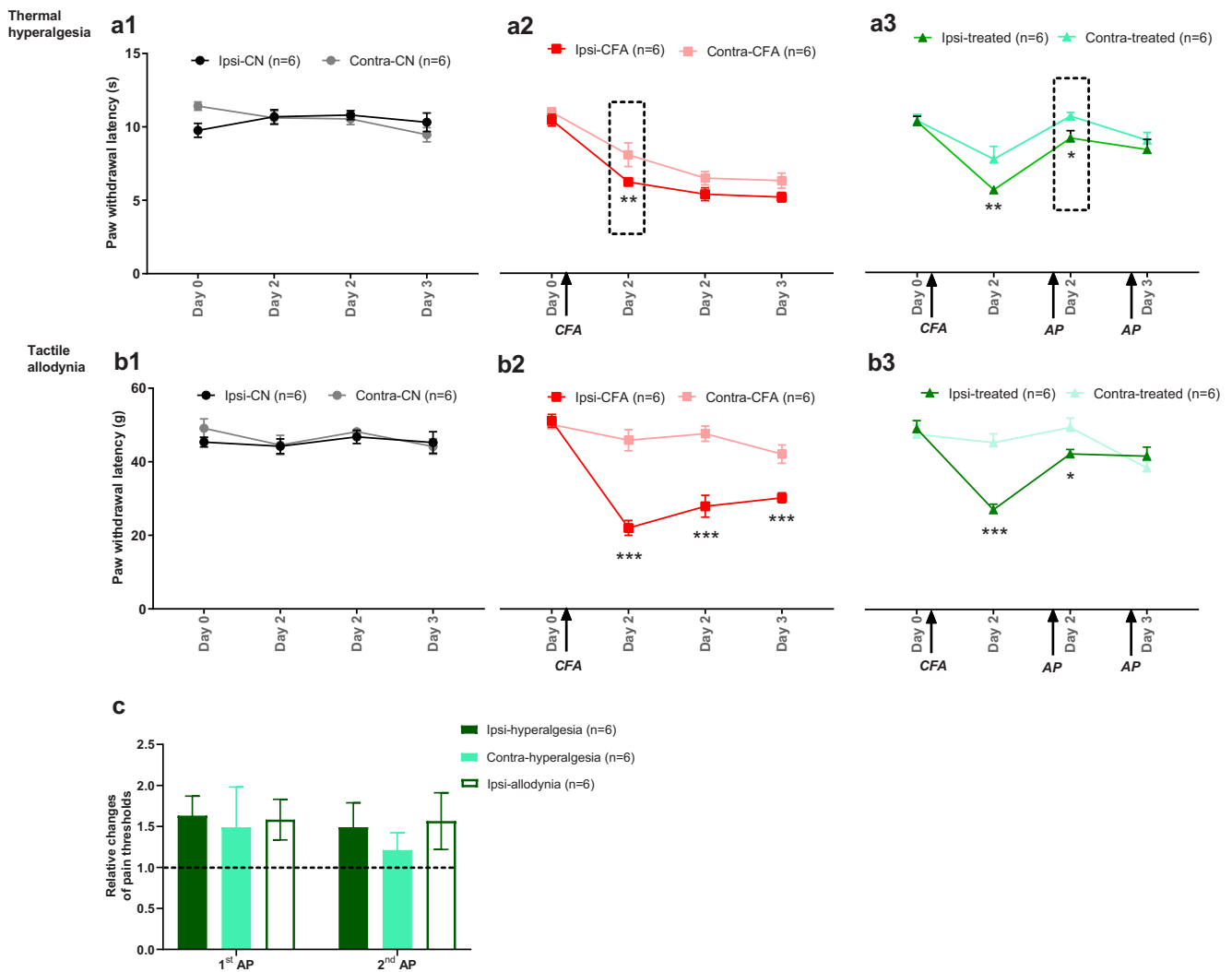


Fig. 3 Comparisons of the pain thresholds between ipsilateral and contralateral hind paws. **a** Thermal hyperalgesia of bilateral paws in control (a1), model (a2), and acupuncture (a3) groups. **b** Allodynia of bilateral paws control (b1), model (b2), and acupuncture (b3) groups. **c** Comparison of the relative analgesia effects of AP on the bilateral hind

paws. In each treatment group (except for the contralateral-allodynia group), the pain threshold after modeling was set as 1. Two sample *t*-test was used. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. contralateral side, respectively

eATP in DRGs, which was further heightened in control DRGs by ARL67156 (100 μ M), a nonspecific inhibitor of ecto-ATPase ($n = 4$, $P < 0.001$) (Fig. 8a). Along with the results that sciatic nerves dramatically hydrolyzed exo-ATP (Fig. 7), we hypothesized that both DRGs and the nerves expressed ecto-ATPase, which contributed the conversion from ATP to ADO.

Various cell-attached or soluble ecto-nucleotidases precisely control eATP levels. Among them, nucleoside triphosphate diphosphohydrolases (NTPDases) family represents the major nucleotide-hydrolyzing enzymes, which degrade ATP to AMP with intermediate formation of ADP [17]. NTPDases family includes NTPDase-1, -2, -3 and -8. We studied NTPDase-2 and -3 in this work because they mainly express in satellite cells and nociceptive-associated small and medium neurons, respectively [18, 19]. Moreover, by directly reducing

eATP level and correspondingly indirectly raising ADO concentration, NTPDase-2 and -3 have been considered to have the function of terminating and regulating pain transmission [18, 20].

Our RT-PCR data revealed that mRNA of NTPDase-2 and NTPDase-3 was expressed in the control ganglia. In injured ipsilateral DRGs, NTPDase-3 was downregulated ($n = 6-8$, $P < 0.05$ vs. blank), on which AP had no effect (Fig. 8b). In contralateral DRGs, NTPDase-2 was greatly upregulated ($n = 6-8$, 2.3 times more than blank, $P < 0.001$ vs. blank), which was further potentiated by AP ($n = 6-8$, 8.9 times more than control, $P < 0.001$ vs. CFA) (Fig. 8c). The expression of contralateral NTPDase-2 in the CFA ($n = 6$, $P < 0.001$) and AP-treated groups ($n = 6-8$, $P < 0.001$) was significantly higher in comparison with ipsilateral side (Fig. 8d1, d2).

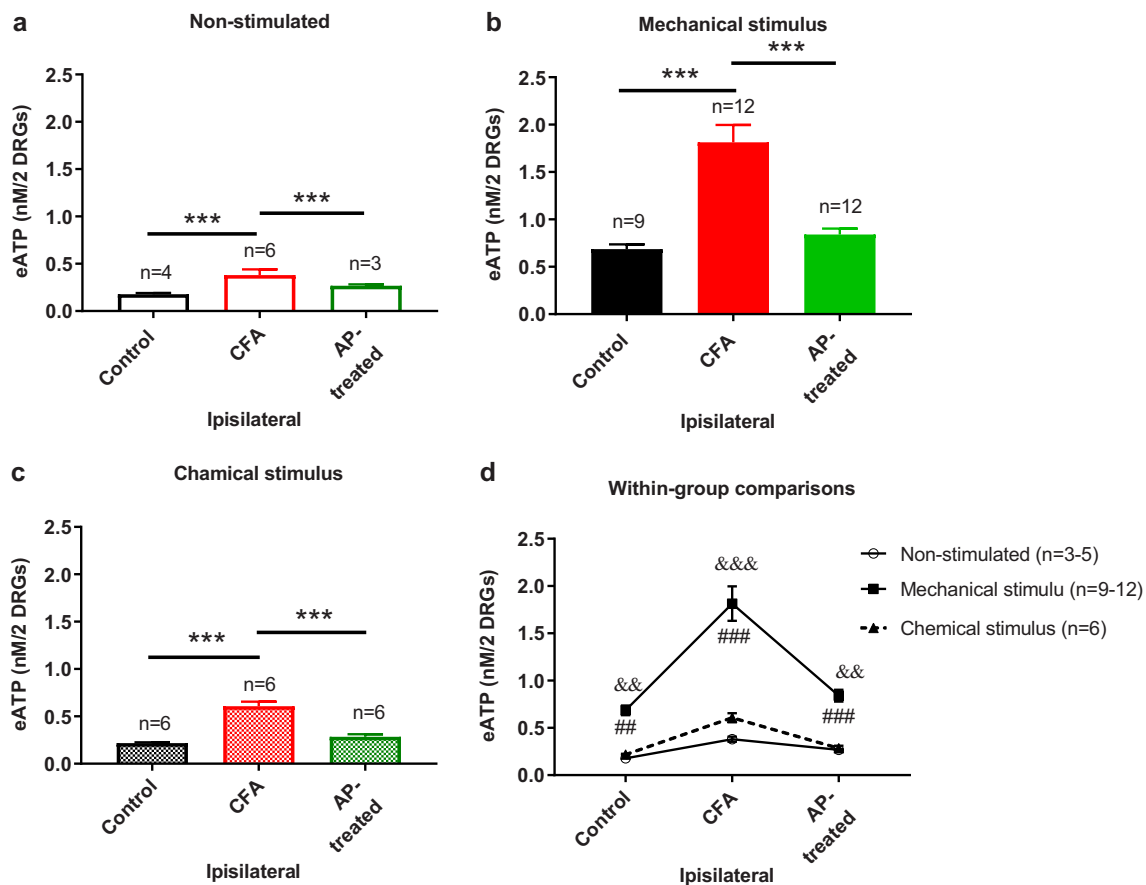


Fig. 4 AP reversed increased eATP levels of injured ipsilateral lumbar DRGs. Ipsilateral lumbar 4-5 DRGs were excised from each group after 2nd AP. **a** Ipsilateral eATP levels assessed from each group in non-stimulated control situation ($n = 3-5$). **b** eATP levels in response to mechanical stimulus which was performed with air bubbles ($n = 10-$

12). **c** eATP levels in response to 100 mM KCl-stimulus ($n = 6$). **d** Within-group comparison of eATP levels based on the data in **a-c**. One-way ANOVA and LSD test were used. $***P < 0.001$. $\&\&P < 0.01$ and $\&\&\&P < 0.001$ vs. non-stimulated, respectively. $\#\#\#P < 0.01$ and $\#\#\#\#P < 0.001$ vs. chemical stimulus, respectively

Regarding sciatic nerve, NTPDase-2 and -3 were also equally expressed in the bilateral control group. The expression of ipsilateral NTPDase-2 was upregulated in the CFA group ($n = 6-8$, $P < 0.01$ vs. blank) but was suppressed by AP treatment ($n = 6-8$, $P < 0.05$ vs. CFA group) (Fig. 9a). Differently, NTPDase-3 was not influenced in the CFA group but was significantly increased in the AP-treated group ($n = 6-8$, $P < 0.001$ vs. CFA) (Fig. 9a). Contralateral side had same responses ($n = 6-8$, $P < 0.001$ vs. CFA group) (Fig. 9b). Comparing the bilateral NTPDases expressions in sciatic nerves (Fig. 9c1, c2), ipsilateral NTPDases-2 in the CFA group was significantly higher than contralateral ($n = 6$, $P < 0.01$). While, this phenomenon was opposite in the AP group ($n = 8$, $P < 0.01$).

Figure 10 demonstrated the comparisons of the NTPDase-2 and -3 expressions between DRGs and sciatic nerves. The changes of NTPDase-2 expression were inconsistent. On the ipsilateral side, sciatic nerves had more NTPDase-2 mRNA than ganglia in the CFA group ($n = 6-8$, $P < 0.001$) (Fig. 10a1). On the contrary, contralateral DRGs expressed more NTPDase-2 than nerves both in the CFA ($n = 6-8$, $P < 0.001$) and in the AP-treated group ($n = 6-8$, $P < 0.001$) (Fig. 10b1).

The changes of NTPDase-3 expression were consistent between bilateral. NTPDase-3 mRNA was significantly higher in treated nerves than that in treated ganglia ($n = 6-8$, $P < 0.001$) (Fig. 10a2, b2).

Discussion

AP relieved bilateral pain hypersensitivity induced by unilateral inflammation

This work unveiled that unilateral ankle arthritis caused the local pain hypersensitivity and the thermal hyperalgesia in bilateral side (Fig. 2a-c). The contralateral response is a dramatic phenomenon of central sensitization [21], which is due to the sensitization of the spinal and supraspinal nerve system [22]. Such dramatic secondary hyperalgesia phenomenon has been reported in capsaicin-induced pain in animal [23] and human [24] tests.

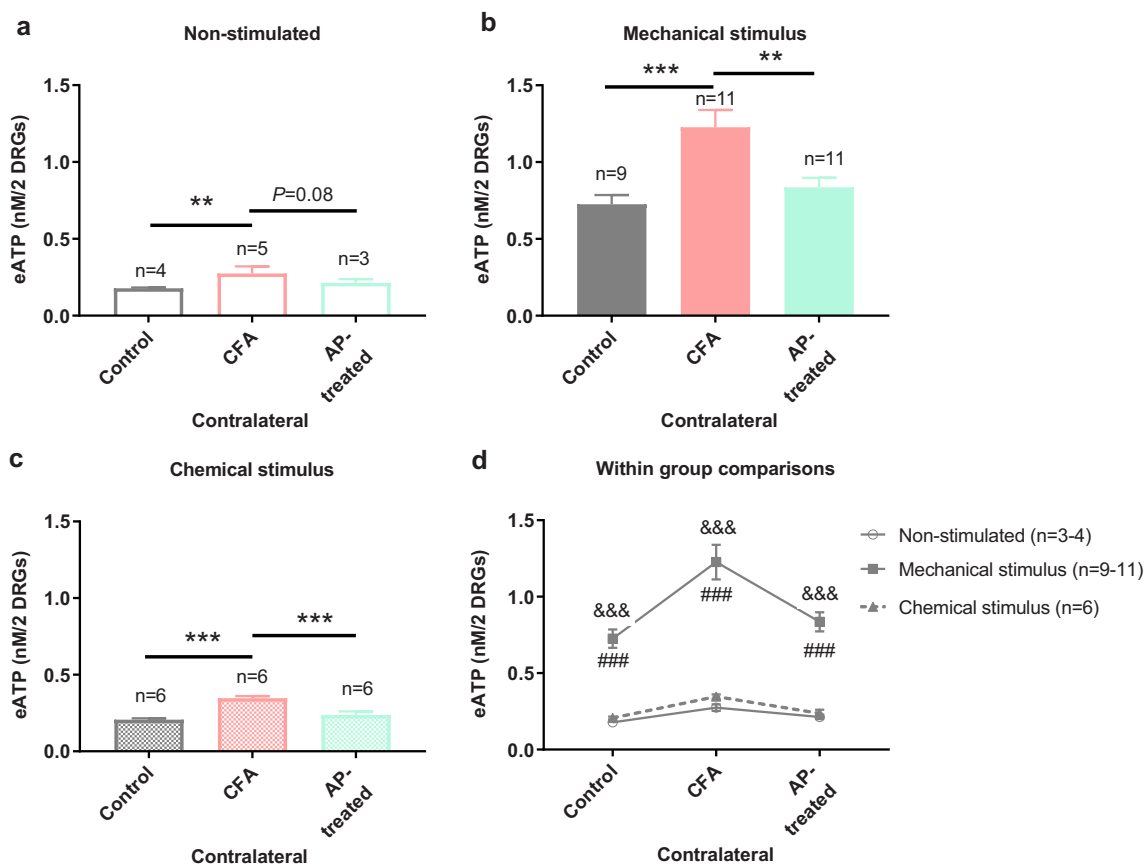


Fig. 5 AP reversed increased eATP levels of injured contralateral lumbar DRGs. Ipsilateral lumbar 4-5 DRGs were excised from each group after 2nd AP. **a** Contralateral eATP levels measured from each group in control situation ($n = 3-5$). **b** eATP levels in response to mechanical stimulus were performed with air bubbles ($n = 9-11$). **c** eATP levels in response to

100 mM KCl-stimulus ($n = 6$). **d** Comparison of eATP levels within groups based on the data in **a-c**. One-way ANOVA and LSD test were used. $**P < 0.01$ and $***P < 0.001$, respectively. $\&\&\&P < 0.001$ vs. each baseline values. $\#\#\#P < 0.001$ vs. chemical stimulus

Although thermal hyperalgesia and allodynia caused by unilateral inflammation were more severe in the ipsilateral paw than in the contralateral one (Fig. 3a2, b2), AP treatment had a similar analgesic efficacy on them (Fig. 3c) indicating AP analgesia has non-specific systemic mechanism. Moreover, the consistent changes of exo-ATP hydrolysis of bilateral sciatic nerves (Fig. 7) also confirm this point. In the field of AP studies, it is common to exert AP in the

contralateral acupoints to treat a given disease, which is referred to as “Juci technique” and has been proven to have equal or even higher anti-nociception effect than ipsilateral AP [25, 26]. Differently, our current work found that ipsilateral AP had an equal analgesia effect on the contralateral secondary hyperalgesia (Fig. 3c). But both events implied that spinal or supraspinal levels are involved in the AP analgesia mechanism. Such non-specific systemic effect might be

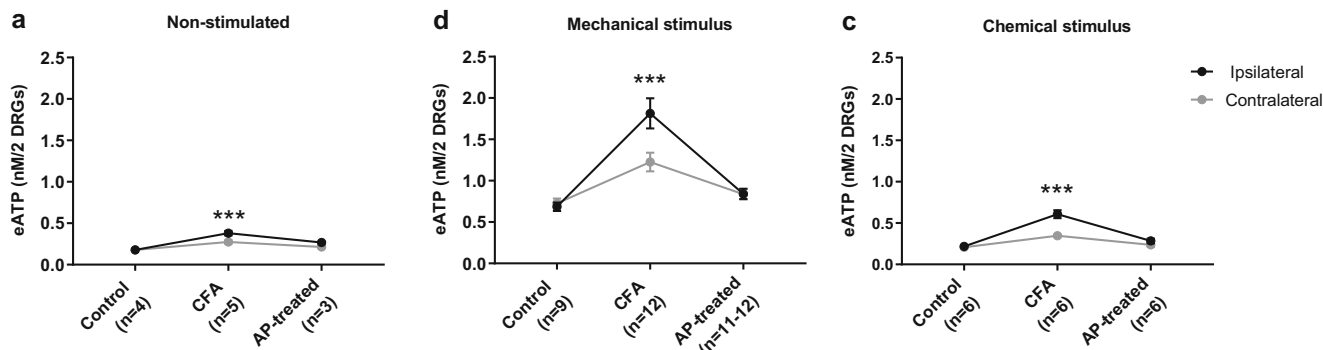


Fig. 6 Comparisons of eATP levels between bilateral DRGs in response to external stimuli. **a** eATP level in the absence of stimuli. **b, c** eATP levels in response to mechanical and chemical stimulus, respectively. Two sample t -test was used. $***P < 0.001$ vs. contralateral DRGs

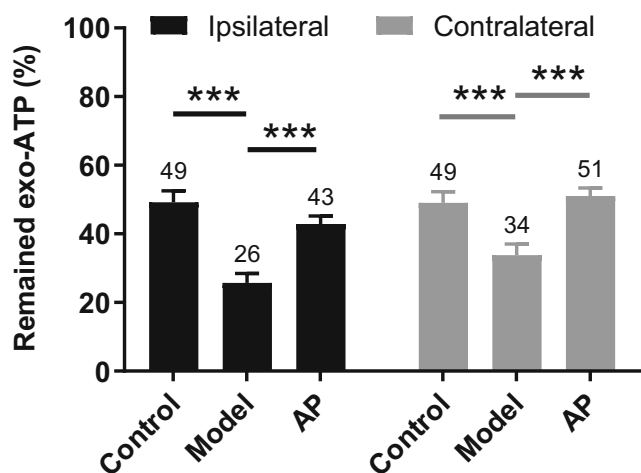


Fig. 7 Exo-ATP hydrolysis of bilateral sciatic nerves in the process of acupuncture-analgesia. The number on the top of each column was the percentages of the remained ecto-ATP. One-way ANOVA and LSD test were used. *** $P < 0.001$

attributed to the involvement of multiple brain areas and various neurochemical substrates in AP analgesia [2, 4].

Besides, AP also has specific anti-nociceptive effect, mainly when acupoints and pain areas are innervated by the same or adjacent spinal segments. According to the principle of spinal segmental innervation, signals from acupoints and pain sites interact in the spinal cord dorsal

horn [2]. This may explain why reversal effect of AP on eATP in ipsilateral ganglia was greater than that in contralateral ganglia (Fig. 6).

Inflammation modulated eATP levels of innervated DRGs and sciatic nerves

ATP can release from neurons somata [14], nerves [27], and nerve endings [15]. Excessive eATP has been found in lumbar DRGs that are injured by neuropathic pain [12]. Our work for the first time revealed that acute inflammatory-injured DRGs also resulted in excessive eATP (Figs. 4a and 5a).

We found that mechanical stimulus dramatically increased eATP level of DRGs of each group, especially the CFA group (Figs. 4d and 5d). Ganglia contain primary somatosensory neurons that have similar physiological properties with nerve endings, including ATP release [14, 15], purinoceptors expression [28], and nucleotidases expression [20]. Thus, the changes in DRG mechanosensitivity may partially reflect the mechanosensitivity of their peripheral nerve endings that innervate the hind paw and ST36 acupoint. But the excessive mechanosensitive of contralateral DRGs cannot explain the normal touch sensation of contralateral hind paw in the CFA group. An alternative explanation may be the tenderness of ST36 acupoint. According to acupoint sensitized theory, the acupoints become activated and sensitized when the body is

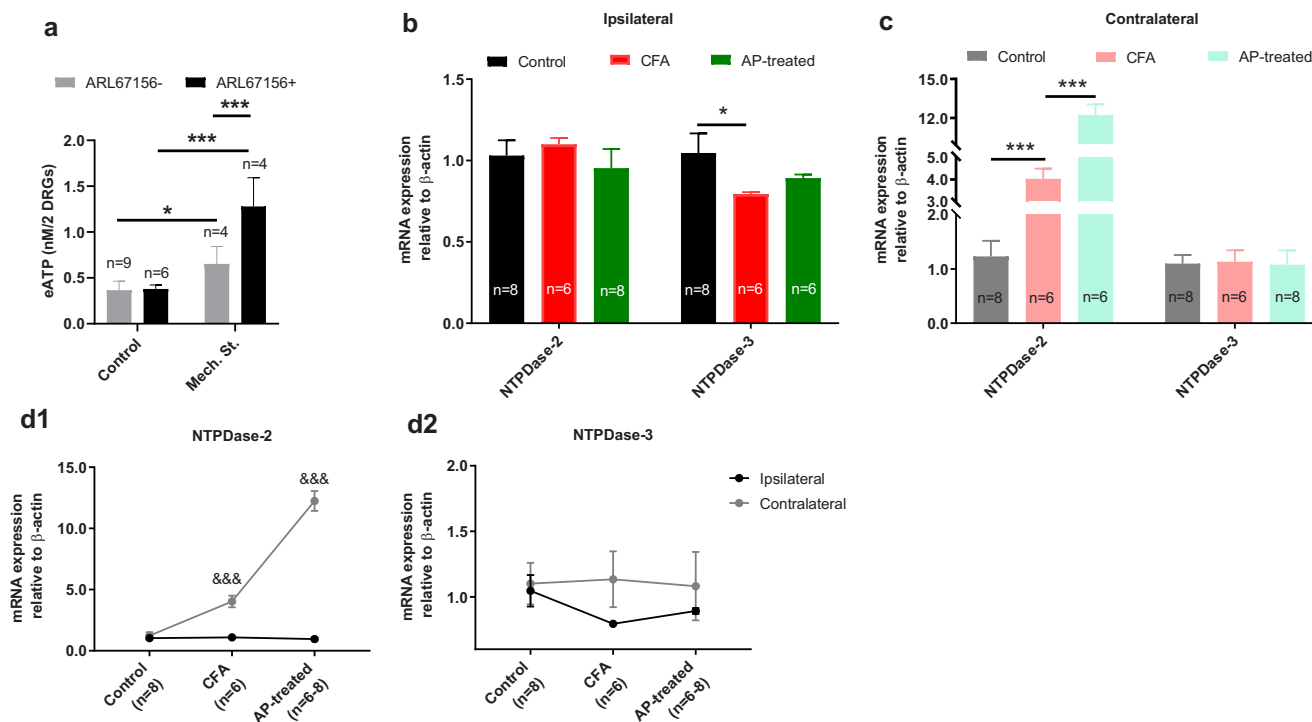


Fig. 8 Changes of NTPDase-2 and NTPDase-3 mRNA expression in bilateral DRGs in the process of AP analgesia. **a** The effect of ARL67156 (100 μ M) on eATP levels of ipsilateral control DRGs ($n = 4-9$). **b, c** NTPDase-2 and -3 mRNA in ipsilateral and contralateral L4-5 DRGs from each group ($n = 6-8$). **d1, d2** comparison of NTPDase-2 and -

3 expression between ipsilateral and contralateral DRGs isolated from each group ($n = 6-8$). One-way ANOVA and LSD test were used in **a-c**. Two sample t -test was used in **d1** and **d2**. *** $P < 0.001$; &&& $P < 0.001$ vs. ipsilateral DRGs

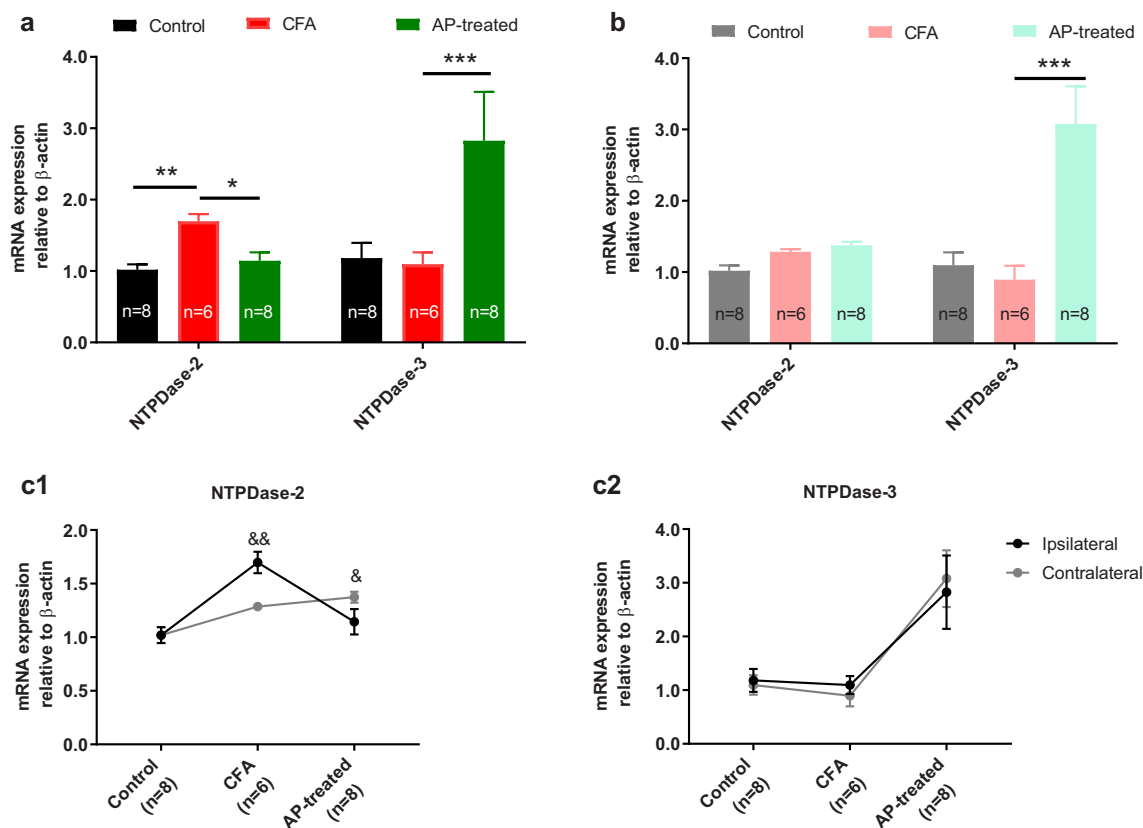


Fig. 9 Modulation of NTPDase-2 and NTPDase-3 mRNA of sciatic nerves by ankle inflammation and acupuncture ST36. **a**, **b** NTPDases expressions in ipsilateral and contralateral sciatic nerves of rats in blank model, and treatment groups, respectively. **c1**, **c2** Comparison of NTPDases-2 and -3 expression between ipsilateral and contralateral

sciatic nerves isolated from different groups. One-way ANOVA and LSD test were used in **a** and **b**. Two sample *t*-test was used in **c1** and **c2**. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively. & $P < 0.05$ and && $P < 0.01$ vs. ipsilateral nerves, respectively

suffering from some corresponding diseases [29]. The sensitized acupoints manifest in different forms, for example, expansion of the receptive field and heat sensitization. Tenderness is also included [30, 31]. These sensitized acupoints distribute not only in local areas but also in distal areas along the corresponding meridians or bilateral sides of the spine [29]. This hypothesis still needs to be evidenced by determining the touch sensations of bilateral acupoints in future.

The expression of purinoceptors is upregulated in DRGs injured by peripheral neuropathic or inflammatory pain or visceral pain, including P2X₃ (P2X_{2/3}), P2X₇, and P2Y_{1, 2, 6} receptors [3, 4]. Our findings about excessive eATP of inflammatory-injured DRGs were an important complementation for enhancing purinergic signaling during pain process. Similar study had unveiled that in the neck incision pain model, eATP level together with the expression of P2X₇ receptors was upregulated in the spinal cord [32].

Regarding sciatic nerves, we unexpectedly uncovered that normal nerves had strong power to hydrolyze exo-ATP (Fig. 7). It has been reported that non-myelinating Schwann cells of rat sciatic nerves express nucleotidases [33]. Electric field

stimulation (8 Hz, 60 s) can induce sympathetic nerves to release soluble nucleotidases [27]. We observed that such hydrolysis capacity was further heightened when injured (Fig. 7). More exo-ATP hydrolysis will result in less activation of pain-related P2XRs and accumulation of anti-nociception-related ADO. We wonder whether it is a self-protective action exerted by nerve system during pain process, similarly to the study that opioid receptors cluster in the CFA-injured sciatic nerves [34]

AP modulated eATP levels of innervated DRGs and sciatic nerves

Compared with purinoceptors, their ligands are less studied in AP analgesic mechanism. To date, EAP has been reported to further upregulate the increased eATP content in the dorsal part of the cervical-spinal cord caused by neck incision in rats [32]. AP markedly increases extracellular ADO in acupoint of human [35] and rodents [36, 37]. We suppose that this ADO accumulation may attribute to eATP hydrolysis. The present work uncovered that AP treatment restored CFA-induced excessive eATP levels (Figs. 4d and 5d). Reducing eATP might

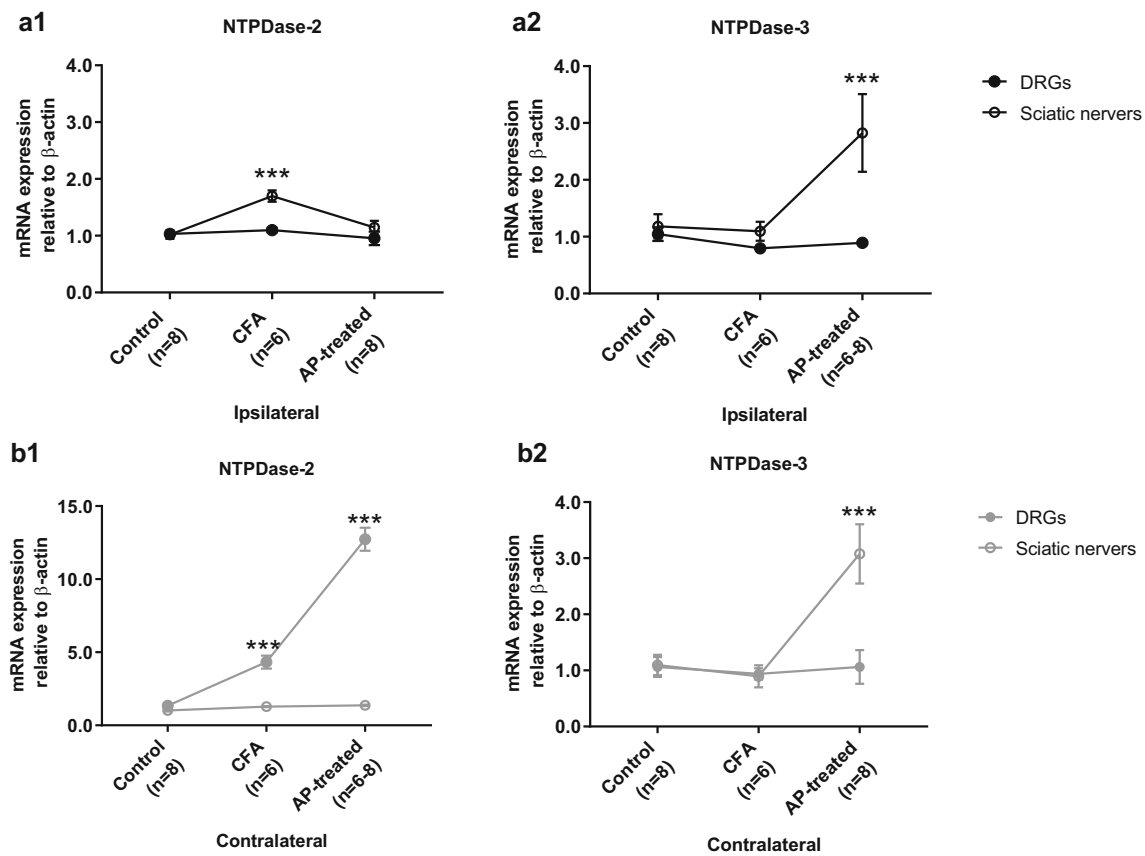


Fig. 10 Comparison of NTPDases mRNA expression between bilateral DRGs and sciatic nerves. **a1, a2** Comparisons of mRNA expression of ipsilateral NTPDase-2 and -3, respectively. **b1, b2** Comparison of mRNA

expression of contralateral NTPDase-2 and -3, respectively. Two sample *t*-test was used. *** $P < 0.001$ vs. DRGs

be another important potential mechanism of AP analgesia, supplemented with downregulation of purinoceptors.

Compared with DRGs, AP-regulated sciatic nerves are less studied. As reported, in CFA-induced ankle arthritis model of rats, AP in ST36 markedly increases the discharges of the sciatic nerves [38]. In injured sciatic-nerve model of rats, EAP in ST36 and GB30 (Huantiao acupoint) change the electrical physiological properties of sciatic nerves, upregulate the expression of some factors, including brain-derived nerve growth factor, nerve growth factor, and growth associated protein-43, and increase the proliferation of the Schwann cells [39]. In the current work, AP retreatment restored the capacity of exo-ATP hydrolysis in sciatic nerves, which was previously potentiated by injured (Fig. 7). As we discussed above, higher exo-ATP hydrolysis in the CFA group may hint at a self-protective effect. Then, reversing exo-eATP hydrolysis through AP may relieve this defense to facilitate body homeostasis. AP, to some degree, enhances the ability of human body to self-medicate by facilitating physiological homeostasis, as a regulator of robustness, to treat model rats.

As most other studies, we examined eATP level after bulk equilibration in extracellular samples with the L-L reagent, which greatly underestimate the true ATP concentration in

the pericellular environment because of the dilution by external buffer and its rapid degradation by ecto-nucleotidases. Recent studies have suggested that true levels of eATP at the plasma membrane are underestimated by >20-fold with bulk phase measurements [40]. Thus, in situ, the changed eATP in our work was enough to activate more P2X receptors. Moreover, due to the presence of nucleotidase in DRGs and sciatic nerves [18, 19], more ADP-sensitive P2YR and P1 receptors were involved.

AP modulated NTPDases mRNA expression in DRGs and sciatic nerves

Besides physiological release, hydrolysis is another main pathway to control eATP. When ATP is released from nerves as a transmitter, there is a concomitant release of nucleotidases that rapidly degrade ATP sequentially to ADP, AMP, and ADO, thereby terminating the action of ATP [27]. In rat cortical astrocytes, the presence of exo-ATP (1 mM) can fasten the hydrolysis of ATP and ADP via increasing the expression of NTPDase-2 and Nt5e [41]. As the major ecto-ATPase, NTPDases hydrolyze ATP into AMP with intermediate formation of ADP [17]. Thus, they can directly downregulate the

activity of P2X receptors, and meanwhile, indirectly upregulate the functions of certain P2Y or P1 receptors by collaborating with some ecto-AMPase, for example, ecto-5'-nucleotidase (Nt5e) [18]. So far, behavior evidence in animal tests has corroborated that prostatic acid phosphatase (PAP) [42] and Nt5e [43, 44], hydrolyzing ATP and AMP into ADO, respectively, have anti-nociceptive effects at spinal cord level via activation of A1 receptors. Despite the lack of behavioral evidence, histomorphometric and histochemical studies have observed that NTPDase-3 is mainly expressed on nociceptive-associated small and medium neurons in sensory ganglia [18, 19] and co-localized with markers of nociceptors [18]. These findings suggest that NTPDase-3 could be a potential target for modulating pain sensation. Differently, NTPDase-2 was mainly expressed in satellite cells in DRG [18] and Schwann cells around sciatic nerves [33]. In sensory ganglia, except for P2X₇, all P2X and P2Y_{1, 2, 4, 11} subtypes are found in sensory neurons [7, 8, 45]. In sciatic nerves, P2X₇, P2Y₁, P2Y₆, and P2Y₁₁ receptors are expressed [8, 46]. Hence, the existence of NTPDases will change the activation of these subtypes.

In the current work and our previous studies [13, 16], the effects of ARL67156 indicated the existence of ecto-ATPase in rats' DRGs, which was confirmed by the RT-PCR. But there were great inconsistent changes of NTPDases between ipsi- and contra-lateral peripheral nervous system, especially NTPDase-2 in DRGs (Fig. 8b, c). The asymmetric changes of NTPDases between ipsi- and contra-lateral (Figs. 8 and 9) might be another underlying distinct mechanism in peripheral nervous system between the conventional and contralateral AP, which is complementary to their different central mechanism [26, 47]. The inconsistent responses between bilateral DRGs and bilateral sciatic nerves can only partly but not fully explain eATP changes of ganglia (Figs. 4a and 5a) and nerves (Fig. 7). But, in general, AP had potentiating effects on NTPDase-2 expression in contralateral DRGs (Fig. 8d1) and NTPDase-3 expression in bilateral sciatic nerves (Fig. 9c2), which are expected to reduce the amplitude and duration of ATP-related pain signal transmission. Of course, these ideas still need further exploration to confirm.

To date, there is no clear evidence of AP regulatory role in ecto-nucleotidase. The only clue is that in mouse models of acute and chronic pain, injection of PAP into Weizhong acupoint (BL40) has anti-nociceptive effects similar to AP treatment, which lasts up to 6 days following a single injection [48].

Conclusions

Unilateral AP could play anti-nociceptive effects on bilateral hypersensitivity, which is accompanied by reversing the increased eATP levels of bilateral injured lumbar DRGs and restoring the heightened exo-ATP hydrolysis of bilateral

injured sciatic nerves. Additionally, the nucleotidases NTPDase-2 and -3 mRNA were modulated in the process of AP analgesia.

Abbreviations ADO, Adenosine; ADP, Adenosine diphosphate; AMP, Adenosine monophosphate; ARL67156, 6-N,N-Diethyl-β-γ-dibromomethylene-D-adenosine-5'-triphosphate; ATP, Adenosine triphosphate; CFA, Complete Freund's Adjuvant; DRG, Dorsal root ganglia; EAP, Electric acupuncture; eATP, Extracellular ATP; exo-ATP, Exogenous ATP; L-L, Luciferin-luciferase; Nt5e, Ecto-5'-nucleotidase; NTPDases, Nucleoside triphosphate diphosphohydrolases; P2 receptors, Purinergic receptors; PAP, Prostatic acid phosphatase; PS, Physiological solution; PWL, Paw withdraw latency; PWT, Paw withdraw threshold

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Code availability Not applicable.

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Data availability The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval All experimental protocols were approved by the Animal Care Committee of Shanghai University of Traditional Chinese Medicine (Shanghai, China) (No SZY201807008).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflicts of interest The authors declare no competing interests.

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