Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2023**; 28(1): 1
https://doi.org/10.31083/j.fbl2801001

Original Research

Mast Cell-Associated Serotonin in Acupoint Contributes to Acupuncture Analgesia in Arthritis Rats by Mediating ATP Release

Bing-Rong Li[1], Yu-Jia Li[2], Jing-Wen Xu[2], Meng Huang[3], Li-Na Wang[2,3,*], Di Zhang[1,*], Xue-Yong Shen[2,3], Guang-Hong Ding[1]

[1]Shanghai Key Laboratory for Acupuncture Mechanism and Acupoint Function, Department of Aeronautics and Astronautics, Fudan University, 200433 Shanghai, China
[2]School of Acupuncture-Moxibustion and Tuina, Shanghai University of Traditional Chinese Medicine, 201203 Shanghai, China
[3]Shanghai Research Center for Acupuncture and Meridians, 200433 Shanghai, China
*Correspondence: lnwang@shutcm.edu.cn (Li-Na Wang); dizhang@fudan.edu.cn (Di Zhang)
Academic Editor: Graham Pawelec
Submitted: 3 September 2022 Revised: 10 November 2022 Accepted: 28 November 2022 Published: 9 January 2023

Abstract

Background: The activation of subcutaneous mast cells (MCs) helps to trigger the analgesic effect induced by acupuncture (AP), a traditional oriental therapy, that has been gradually accepted worldwide. This work aimed to reveal whether the serotonin (5-hydroxytryptamine, 5-HT) released from MCs plays an important role in this process, which has a controversial effect in the mechanism of pain. **Methods**: *In vivo* tests, a 20-min session of AP was applied at Zusanli acupuncture point (acupoint) of acute ankle arthritis rats. Pain thresholds of the injured hindpaw were assessed to reflect the pain state, and the targeting substances in the interstitial space of the treated acupoint were sampled by microdialysis. *In vitro* experiments, exogenous 5-HT (exo-5-HT) was introduced to mediate adenosine triphosphate (ATP) release from cultured MCs. **Results**: Needling promoted 5-HT accumulation at the Zusanli acupoint, which was prevented by sodium cromolyn. AP's analgesic effect was suppressed by the inhibition of 5-HT receptors at the acupoint, especially 5-HT_{1A} subtype. *In vitro* tests, mechanical perturbation mimicking needling stimulation induced MCs to release 5-HT. 1 μM and 10 μM of exo-5-HT facilitated ATP release, which was restrained by blocking of 5-HT_1 receptors rather than 5-HT_3 receptors. As 5-HT, ATP and adenosine were also transiently accumulated in the treated acupoint during needling. Promoting ATP hydrolysis or activation adenosine A1 receptors duplicated AP analgesic effect. Finally, the inhibition of ATP receptors by suramin or pyridoxal phosphate-6-azo tetrasodium salt hydrate (PPADS) prevented AP analgesic effect. **Conclusions**: Our results suggest that MC-associated 5-HT release at acupoints contributes to AP analgesia, and the mediation of ATP secretion through 5-HT_{1A} receptors might be the underlying mechanism at play. ATP could facilitate adenosine production or the propagation of needling signals.

Keywords: acupuncture analgesia; mast cells; 5-HT; 5-HT_{1A} receptor; ATP

1. Introduction

Alongside subcutaneous tissue and skeletal muscles, the skin is an important structural layer of acupuncture points (acupoints), which are special sites on the body surface that receive a variety of physical stimuli to modulate functional disorders of inner organs. Our researches and some other related studies have shown that subcutaneous mast cells (MCs) are present at greater densities in acupoints, and their activation plays an initiating role in the mechanism of acupuncture (AP) [1], an ancient oriental therapeutic approach gradually becoming more accepted worldwide. During AP, fine needles are inserted into certain acupoints and subsequent manipulations generate mechanical stimulation on acupoint, 240–280 mN in force, and 10–15 mN $\times$ mm^{-1} in torque [2]. These signals are transmitted to the wider and deeper space by subcutaneous collagen fibers twisting around the needles [3]. Our studies *in vitro* reveal the mechanosensitivity of MCs and tests performed *in vivo* have uncovered such characteristic contributes to AP analgesia [4,5]. Additionally, MCs can also be activated by biological agents. Substance P (SP) and calcitonin gene related peptide (CGRP) released from the nerve terminals due to needling stimulation can induce adject MCs to degranulate [6].

Our previous work *in vivo* had shown that MCs-released histamine was involved in the trigger mechanism of AP analgesic effect via activating H1 receptors [4]. In the current work, we wondered whether serotonin (5-hydroxytryptamine, 5-HT) was also involved in this process, which is another endogenous substance exported from subcutaneous MCs [7]. In rat acupoints, 5-HT-immunopositive MCs are present in subcutis and dermis, and their degranulation occurs in response to AP [8,9]. 5-HT has a controversial effect in pain mechanism, in which pain or analgesia relies on which subtype of 5-HT receptors (5-HTRs) is activated [10]. Considering the tissue components of acupoints, 5-HT_{1A}R and 5-HT_3R might be expressed [11], and they mediate the sustained and transient pain processing on rat neurons, respectively [12]. Therefore, the current work tried to address whether MCs-

released 5-HT also contributes to AP-analgesia via binding to some 5-HT_1Rs or 5-HT_3Rs subtypes.

5-HT-related signaling interacts with purinergic signals that are widely involved in pain mechanism [13]. Adenosine is a vital mediator for anti-nociceptive effect in AP analgesia via activating adenosine A1 receptors (A1Rs) [14]. Adenosine triphosphate (ATP), as the precursor of adenosine, had been found to mechano-sensitively release from MCs *in vitro* [5,15]. Our recent publication confirmed *in vivo* the needling-induced interstitial ATP (inters. ATP) accumulation in the interstitial space of acupoint [16]. In type II cells of rat's carotid body, exogenous 5-HT (exo-5-HT) increases $[Ca^{2+}]$i partially via activating pannexin-1 (Panx-1) channels, implying 5-HT might facilitate ATP release [17]. Hence, we also wondered whether 5-HT is involved in AP-analgesia by mediating ATP release.

Our results verified that in acute adjuvant arthritis (AA) rat model, MCs-associated 5-HT accumulated in treated acupoint and contributed to AP-analgesia. The underlying mechanism might be that mechanosensitive 5-HT release promote ATP secretion and initiate the downstream signalings.

Although these considerable basic studies have been executed to explore the underlying mechanisms, it is still not fully understood. A better understanding of how a fine needle to trigger the anti-nociceptive signals at the acupoint would shed light on the initiation mechanism of AP and provide guidance for proper clinical operation.

2. Materials and Methods

2.1 Animals

SPF-grade male Sprague-Dawley (SD) rats weighing 200 $\pm$ 20 g were used. The rats were purchased from Zhejiang Viton Lihua Experimental Animal Technology Co., Ltd., and maintained at the Animal Experimental Center of Shanghai University of Traditional Chinese Medicine. We performed the animal experiments in accordance with the procedures approved by the Animal Experimental Center (Animal Ethics No.: PZSHUTCM200911014) and implemented the relevant provisions from the Guidance Suggestions for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of the People's Republic of China.

2.2 Rat Model of AA

We used the rat model of AA established as previously described [16]. Briefly, 50 μL complete Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) was injected into the left ankle joint cavity of rats anesthetized with 1.5% isoflurane. Local swelling and behavioral disability appeared within 24 h.

2.3 AP Treatments

A single course of AP treatment for 20 min was performed at Zusanli (ST 36) acupoint of the affected side of conscious rats. According to our researches and related studies [4], the ST 36 has a desirable anti-nociceptive effect on AA rats. ST 36 is located at the posterolateral side of knee joint, about 5 mm below the fibula capitulum. A stainless-steel needle (0.18 mm $\times$ 13 mm, Cloud Dragon Medical Equipment Company, Wujiang, China) was gently inserted to an approximate depth of 7 mm. AP procedure was dominated by twirling-rotating motion (~100 times/min), supplemented with lifting-thrusting motion (~80 times/min).

2.4 Behavioral Tests

Pain thresholds of the injured hind plantar were determined to reflect the pain levels. Mechanical allodynia (paw withdrawal threshold, PWT) and thermal hyperalgesia (paw withdrawal latency, PWL) were evaluated to determine the pain level. The timeline and methods for behavioral tests was referred to our published work [16,18]. Briefly, CFA was administrated on day 0 and AP was performed 2 days later. Pain thresholds were determined for three times, just before CFA injection, just before AP intervention, and after 30 min-AP treatment. Thermal hyperalgesia was measured 2 h after the mechanical allodynia assessment.

2.5 Reagents and Solutions

All stock solutions were stored at –20 °C and diluted into working solutions to final concentrations when used. Sodium cromolyn (CRO, final concentration: 0.02 g/mL, 20 μL/paw, #C0399, Sigma-Aldrich, St. Louis, MO, USA), a MC stabilizer was pre-injected into the left ST 36 in layers 10 min prior to AP treatment, with half receiving 5 mm subcutaneous injections into the muscle and the other half receiving 2 mm subcutaneous injections under the dermis. Granisetron hydrochloride injection (Gran., 1 mg/mL, 20 μL/paw, National medicine permission number: H20043610) was procured from Guorui Pharmaceutical Co. Ltd. (China National Pharmaceutical Group, Beijing, CN). Methiothepin mesylate (Meth., #T2190, TopScience Co., Ltd., Shanghai, CN) were dissolved in dimethyl sulfoxide (DMSO), and the final concentration was 5 mg/mL, 20 μL/paw. WAY-100635 (WAY., 2 mg/mL, 20 μL/paw, #W108, Sigma-Aldrich, St. Louis, MO, USA), ARL67156 (ARL, 100 μM, 50 μL/paw, #A265, Sigma-Aldrich, St. Louis, MO, USA), 2-Chloro-N6-cyclopentyladenosine (CCPA, 0.04 mg/mL, 20 μL/paw, #C7938, Sigma-Aldrich, St. Louis, MO, USA), Suramin (100 μM, 50 μL/paw, #574625, Sigma-Aldrich, St. Louis, MO, USA) and Pyridoxal phosphate-6-azo tetrasodium salt hydrate (PPADS, 100 μM, 50 μL/paw, #P178, Sigma-Aldrich, St. Louis, MO, USA) were prepared with distilled water. All the agonists and antagonists were pre-injected into the left ST 36 in layers prior to AP treatment. CRO (25 μM), Compand 48/80 (16 μM, #C2313, Sigma-Aldrich, St. Louis, MO, USA), Meth. (100 nM), Gran. (100 nM) and WAY. (100 nM) were introduced to cells 20 minutes prior

to the application of mechanical stimulation or exo-5-HT and were present during the entire process. Preparation of bath solution and 50% hypotonic solution was described in our previous work [5].

2.6 Acupoint Injection

In order to intervene certain corresponding signals at the acupoints, 20–50 μL related reagents were pre-injected into the left ST 36 in layers 20 min prior to AP treatment.

2.7 Microdialysis

The performance of microdialysis technology referred to our published work [19]. Briefly, after rat was anesthetized with 1.5% isoflurane, a linear microdialysis probe (CMA 31, CMA Microdialysis AB, Kista, Sweden) was implanted at a distance of 4–6 mm from ST 36. The dialysis solution, Hank's balanced salt solution (HBSS, Corning Cellgro, Manassas, VA, USA), was controlled by syringe pumps (NE-1000, New Era Pump Systems, Farmingdale, NY, USA) and continuously perfused at a rate of 1 μL/min. Subsequently, rat was restrained in the rat fixator and was gradually awakened from anesthesia. After a 2 h recovery period, microdialysis outflow was collected for 30 minutes per sample.

2.8 Determination of 5-HT, ATP and Adenosine in the Microdialysates

5-HT and ATP in the microdialysate were determined by ELISA kit (HZ-5-HT-RA, Zhen Shanghai and Shanghai Industrial Co., Ltd., CN) and luciferin-luciferase assay (L-L, Sigma-Aldrich, St. Louis, MO, USA), respectively. The procedures were performed according to the manufacturer's instructions. The light emission was measured by a microplate reader (Synergy Mx, BioTek, Winooski, VT, USA). Calibrations were executed before and after each dertermination.

Adenosine levels was performed by high-performance liquid chromatography (HPLC) as previously described [4]. The adenosine standard was checked both before and after the measurement, and the concentrations were 3000 nM, 1000 nM, 300 nM, and 100 nM. The detection results of the animal samples were compared to the standard according the peak areas, and the concentration was calculated.

2.9 Tissue Sectioning and MC Staining

Tissue sectioning and MC Staining referenced to our previous article [4]. Briefly, after the final behavioral test, a 5 $\times$ 5 $\times$ 5 mm specimen of ST 36 was taken and 5 μm paraffin sections were prepared. After staining with toluidine blue, the MCs were dark purple in color. MCs with a blurred boundary, or dispersed granules were counted as degranulated MCs.

2.10 Western Blotting

The performance of western blotting referred to related research [20]. We used rabbit monoclonal antibodies: GAPDH (#ab8245, Abcam, Waltham, MA, USA), 5-HT_{1A}R (#ab85615, Abcam, MA, USA), 5-HT_{1D}R (#ab140486, Abcam, MA, USA), 5-HT_{1B}R (#PA1-41069, Thermo Fisher Scientific, Waltham, MA, USA), 5-HT_{1F}R (#PA5-106542, Thermo Fisher Scientific, MA, USA), Connexin 43 (#71-0700, Thermo Fisher Scientific, MA, USA), Pannexin 1 (#488000, Thermo Fisher Scientific, MA, USA) and $P2Y_1$ (#P6487, Sigma-Aldrich, St. Louis, MO, USA). The proteins were then incubated with peroxidase-conjugated goat anti-rabbit IgG (#7074, Cell Signaling Technology, MA, USA). A visualizer was then used to observe the stripe, and the grayscale value was calculated using Image J v1.8.0 (National Institutes of Health, Bethesda, MD, USA).

2.11 Cell Origin and Culture

The human MC (HMC-1) cell line was kindly provided by Dr J. H. Butterfield (Mayo Clinic) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. Rat basophilic leukemia (RBL-2H3) cell line was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, CN) and cultured in Minimum Essential Medium (MEM) medium. The culture medium was supplemented with 10% Fetal Bovine Serum (FBS), 100 units/mL penicillin and 100 units/mL streptomycin in a 5% CO_2 humidified atmosphere at 37 °C.

2.12 Mechanical Stimulation

Hypotonic shock was introduced to HMC-1 cells to induce degranulation, and tryptase or ATP release. The entire procedure referenced to our article [15]. Briefly, 1–2 mL of HMC-1 cell suspension (0.5–1.5 $\times$ 10^6 cells/mL) was gently introduced into the polycarbonate filter chamber, and superfused with bath solution at 1.3 mL/min. After an equilibration period, hypotonic solution (200 mOsm/Kg H_2O) was introduced to the cells and the perfusate was collected at 1 min. Hypotonic environment is a common method to stimulate cells with force [21]. The cell becomes swollen and its cellular membrane is extended, then the corresponding downstream signals are initiated. In our previous work, we used hypotonic shock to activate mast cells *in vitro* to mimic manual AP [5,15,22]. Tryptase or ATP were assessed by ELISA (#IMM001, Sigma-Aldrich, St. Louis, MO, USA) and luciferase-luciferin assay. For 5-HT release measurements, we stimulated the cells by medium displacement. HMC-1 cells grew in the 96-well plate containing 100 μL culture medium. Half of the medium was gently pipetted up and down ten times with a pipette gun. Subsequently, 5-HT in the cell suspension was qualified by ELISA assay.

2.13 Exo-5-HT Stimulation

For each experiment, RPMI 1640 and MEM medium was used to further dilute 10 mM 5-HT stock solutions to acquire various required concentrations of 5-HT. 5-HT-sensitive ATP released from HMC-1 cells in response to exogenous 5-HT (exo-5-HT, #H9523, Sigma-Aldrich, St. Louis, MO, USA) in the range of 1 nM–10 μM for 15 min. Released ATP levels in the fractions were quantified by L-L assay, as described in 2.8. Based on our preliminary results, when exo-5-HT concentration $\geq$50 μM, cell mortality tended to be above 80%, and the large increase of ATP at this time might be caused by cell death.

2.14 Statistical Analysis

The measurement data are expressed as the mean $\pm$ standard error (SE) values. The data were analyzed using SPSS 25.0 (IBM Co., Armonk, NY, USA). The figures were prepared by GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). Differences among multiple groups were tested with one-way or two-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD.) test. Where only two groups were compared, a two-sample *t*-test was used. When data were non-normally distributed, a Mann-Whitney U test was performed. We considered $p < 0.05$ to be statistically significant.

3. Results

3.1 5-HT Released from MCs in Response to AP and Contributed to Analgesic Effect

We revealed that 30 min-needling caused 5-HT accumulation in the interstitial space of treated acupoint. Inters. 5-HT concentration remarkably increased from 5.5 ng/mL $\pm$ 0.2 ng/mL (time = 0) to 11.4 ng/mL $\pm$ 0.4 ng/mL (t = 30 min) (n = 4, $p < 10^{-15}$) gradually restored to the baseline level (Fig. 1A). Inters. 5-HT amount was significantly potentiated from baseline of 156.8 pg $\pm$ 5.8 pg (-30–0 min) to 252.5 pg $\pm$ 7.2 pg (0–30 min) ($p = 1.2 \times 10^{-12}$, n = 4) (Fig. 1B). Such potentiation effect lasted for at least 120 min. AP-induced 5-HT mobilization was canceled by the presence of CRO (0.02 g/mL, 20 μL), a stabilizer of MCs, which was pre-injected in ST 36 before AP intervention (Fig. 1A,B). These results suggest that needling activate MCs in acupoint to release 5-HT.

Behavioral test revealed that CFA-treated rats suffered from pain hypersensitivity, exhibiting tactile allodynia (PWT) (reduced from 54.9 g $\pm$ 1.1 g to 11.1 g $\pm$ 1.4 g, n = 6, $p = 2.4 \times 10^{-13}$ vs. baseline) and thermal hyperalgesia (PWL) (reduced from 19.6 s $\pm$ 0.8 s to 6.6 s $\pm$ 0.1 s, n = 6, $p = 3.6 \times 10^{-8}$ vs. baseline), but clearly benefited from 30 min-AP at ST 36 (PWT: 43.2 g $\pm$ 2.8 g, n = 6, $p = 2.4 \times 10^{-13}$ vs. before AP; PWL: 15.7 s $\pm$ 1.4 s, n = 6, $p = 1.7 \times 10^{-4}$ vs. before AP) (Fig. 1C,D).

Based on our previous research, the microdialysis recovery percentage was in the range of 25–40% [19]. Considering 20 $\times$ dilution by diffusion and/or convection into the bulk extracellular volume [19,23], the real concentration of 5-HT at the cell surface near the AP site was more than 500 ng/mL (2.8 μM), indicating some 5-HT receptors might be activated [17,24,25]. Considering the expressions and effects in pain processing in acupoints [11,12], we tried to verify the role of 5-HT$_1$Rs and $_3$R. Co-injection of Methiothepin (5 mg/mL, 20 μL), a nonselective antagonist of 5-HT$_1$Rs, and Granisetron (1 mg/mL, 20 μL), a selective antagonist of 5-HT$_3$R, in ST 36 prior to AP suppressed the anti-nociceptive effect of AP on PWT (13.2 g $\pm$ 1.0 g, n = 6, $p = 1.3 \times 10^{-7}$ vs. AP) and PWL (6.3 s $\pm$ 0.7 s, n = 6, $p = 0.0014$ vs. AP) (Fig. 1E,F). Additionally, compared with normal rats, CFA induced more 5-HT mobilization in rats (**Supplementary Fig. 1**), suggesting MCs in acupoint changed dynamically with the ankle arthritis.

AP-induced subcutaneous MCs degranulation is the basis for triggering the analgesic mechanism [3,4], which was confirmed in the current work (Fig. 1G,i). In addition, MCs in the connective tissue slice *ex vivo* prepared from rat ST 36 (Fig. 1G,ii) and cultured HMC-1 cells *in vitro* (Fig. 1G,iii) also degranulated in response to mechanical stimulation of hypotonic shock (200 mOsm/Kg H_2O) that was used to mimic needling stimulation. Determination of the mediator in cellular suspension showed that when HMC-1 cells were perturbed with 50% hypotonic shock, tryptase release, a classic index for MCs degranulation [26], was potentiated from 23.5 ng/mL $\pm$ 2.4 ng/mL of control to 41.5 ng/mL $\pm$ 2.9 ng/mL (n = 10, $p = 0.0004$, vs. Control) (Fig. 1H). In addition, secretion of stress-sensitive molecular ATP and 5-HT were also strengthened (ATP: from 16.1 $\pm$ 1.2 ng/mL of control to 32.1 $\pm$ 3.2 ng/mL, n = 8–10, $p = 0.0001$ vs. Control; 5-HT: from 4.1 ng/mL $\pm$ 0.2 ng/mL of control to 7.4 ng/mL $\pm$ 0.3 ng/mL, n = 7–8, $p = 1.1 \times 10^{-6}$ vs. Control) (Fig. 1H). 5-HT is well known to be released in the manner of degranulation [27]. While, unexpectedly, mechanosensitive ATP release was neither suppressed by CRO nor enhanced by Compand 48/80 (**Supplementary Fig. 2A**), but tryptase release was remarkably affected (**Supplementary Fig. 2B**), indicating that ATP secreted from MCs in a degranulation-independent manner.

3.2 5-HT Induced ATP Release from MCs via Acivation of 5-HT$_{1A}$R

5-HT is reported to induced ATP release from type II cells of the rat carotid body [17]. Mechanosensitive ATP release is discovered in various mammalian cells [21], including MCs [5]. Needling-induced ATP transient accumulation in treated acupoint is supposed as one of the primary steps toward AP-analgesic mechanism by providing the precursor for adenosine [16,19]. Besides primary mechanosensitive ATP release, secondary ATP secretion had been found in MCs in our previous work [15]. Subsequently, we tried to verified whether 5-HT could mediate MCs to release ATP. Fig. 2A shows the dependency of

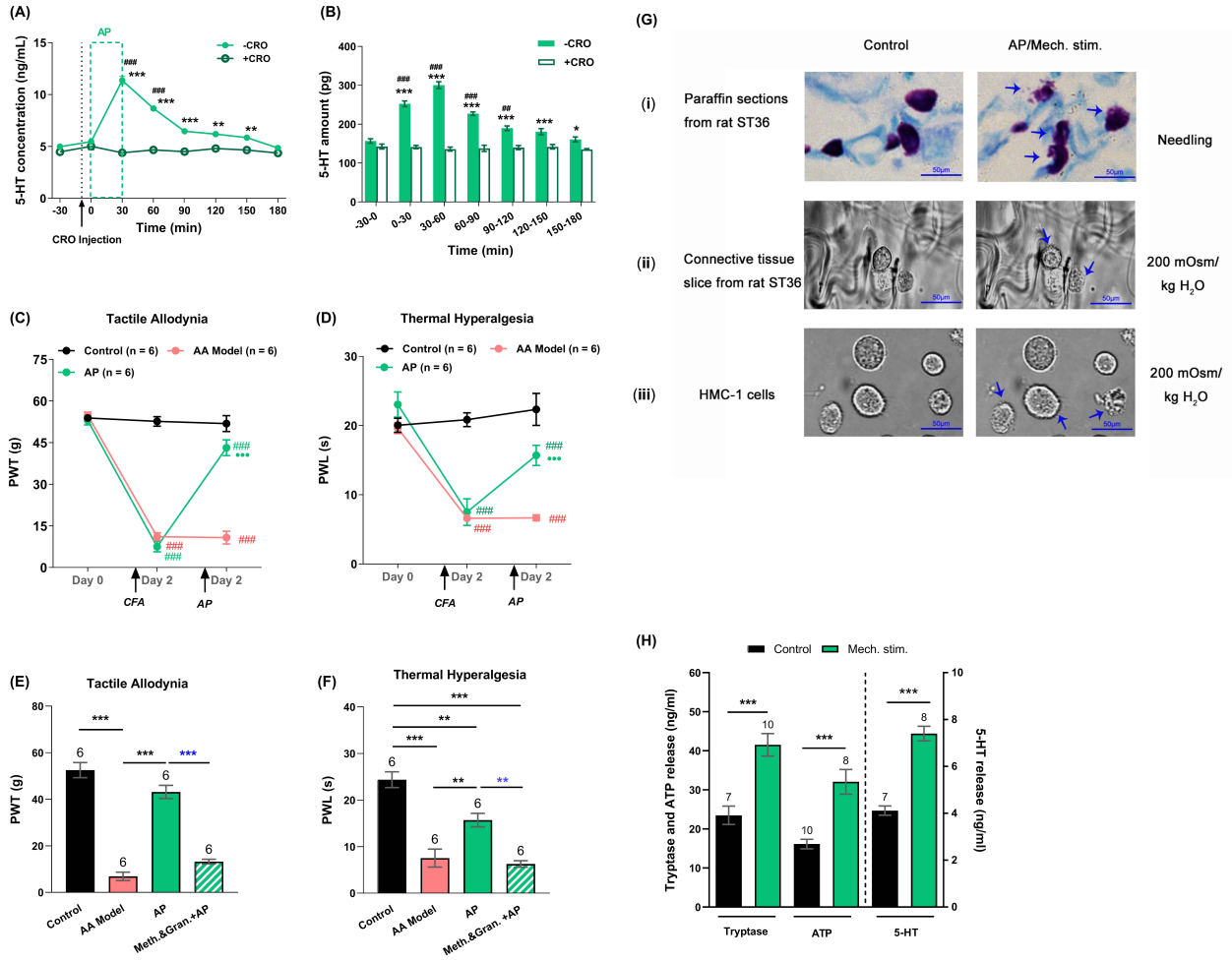

Fig. 1. AP mobilized 5-HT release from MCs in acupoint, which contributed to AP-analgesic effect (n = 4–10, mean ± SE). (A) Time-course of inters. 5-HT concentration in response to needling on AA model rats in the absence and presence of CRO (0.02 g/mL, 20 μL) pre-injected in ST 36 (n = 4). Inters. 5-HT was collected by microdialysis technique and determined by ELISA. ### $p < 0.001$ vs. baseline (time = 0 min); ** and ***, $p < 0.01$ and 0.001 vs. +CRO, respectively. *Two-way ANOVA* and *LSD. test* were used. (B) Changes of inters. 5-HT amount in response to AP in the absence and presence of CRO (n = 4). They were calculated based on the area under the curve in (A). ## and ###, $p < 0.01$ and 0.001 vs. baseline (time = -30–0 min); *, ** and ***, $p < 0.05$, 0.01 and 0.001 vs. +CRO, respectively. (C,D) Effects of AP treatment on CFA-induced tactile allodynia (PWT) and thermal hyperalgesia (PWL) of injured-side hindpaws, respectively. ### $p < 0.001$ vs. baseline (Day 0). ●●● $p < 0.001$ vs. Day 2, before AP. *Two-way ANOVA* and *LSD. test* were used to perform within-group comparisons. (E,F) Effects of pre-co-injection of Methiothepin (Meth.) and Granisetron (Gran.) in ST 36 on AP-relieved PWT and PWL, respectively. Data illustrate the final behavioral tests. ** and ***, $p < 0.01$ and 0.001, respectively. *One-way ANOVA* and *LSD. test* were used to perform between-group comparisons. (G) AP/Mechanical stimulation induced MCs to degranulate. Allows point to degranulated MCs. (i) Paraffin sections prepared from ST36 of AA model group (left) and AP group (right). MCs were stained with toluidine blue and were marked in purple. (ii) Connective tissue slices isolated from ST 36 of normal rat before (left) and after (right) hypotonic shock. (iii) HMC-1 cells before (left) and after (right) hypotonic shock. In (ii) and (iii), the tissue slice and HMC-1 cells were incubated in 200 mOSm/kg H2O-hypotonic solution for 25 min and 15 min, respectively. The plasma membrane of degranulated MCs became rough and the granules scattered around the cells. Scale bar: 50 μm. Mech. stim.: Mechanical stimulation. (H) Release of tryptase, ATP and 5-HT from HMC-1 cells in response to 50% hypotonic shock (n = 7–10). *Unpaired t-test* was performed for two groups' comparison. *** $p < 0.001$. In (E–G), numbers above each column denote sample size (n).

ATP generation on the concentration of exogenous 5-HT (exo-5-HT) (1 nM–10 μM). When the concentration of exo-5-HT reached 1 μM and 10 μM, extracellular ATP level was significantly potentiated from baseline of 15.7 nM ± 2.5 nM to 43.6 nM ± 5.3 nM and 107.3 nM ± 3.4 nM, respectively (Fig. 2A). 5-HT concentration greater than 10 μM was not determined because cells viability was affected (**Supplementary Fig. 3**).

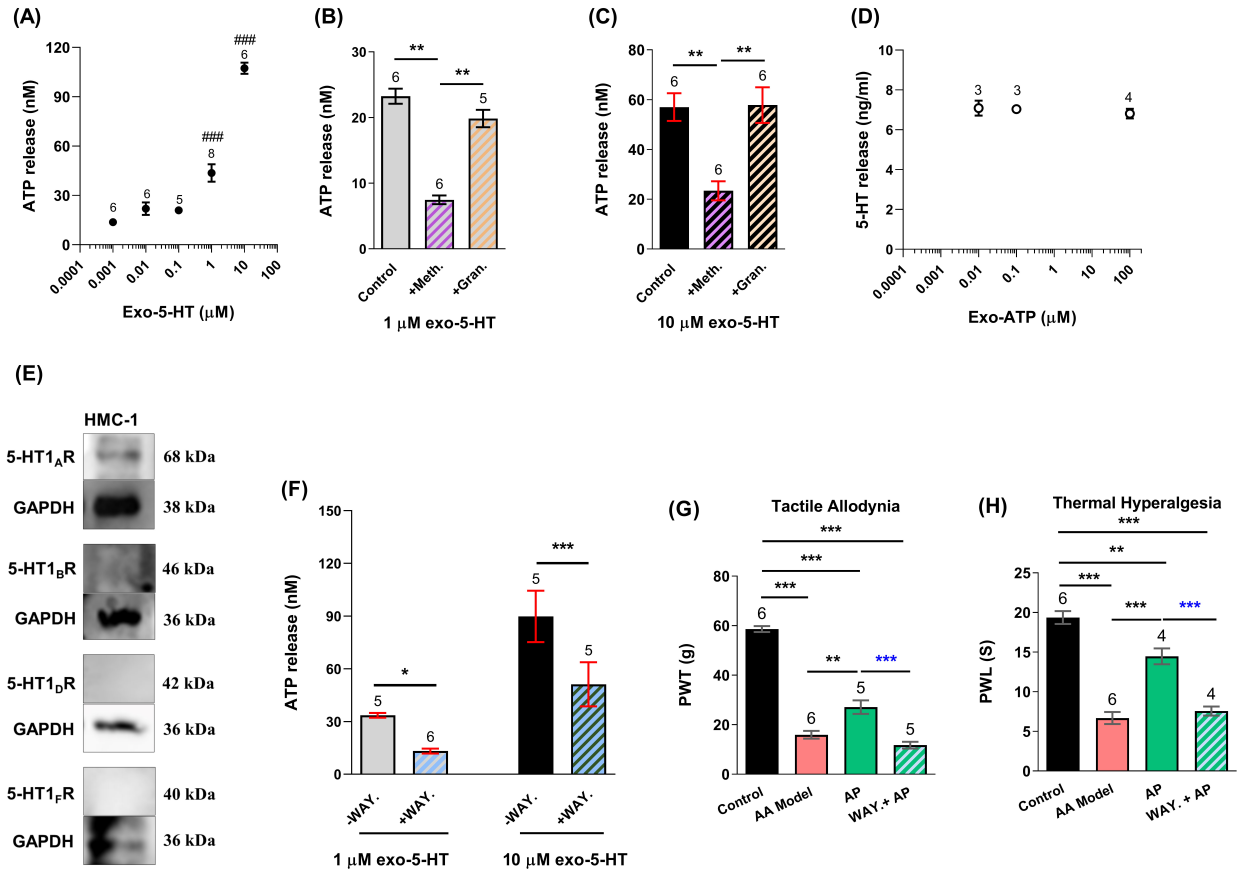

Fig. 2. 5-HT receptors mediated ATP release and contributed to AP analgesia (n = 3–8, mean ± SE). (A) Concentration-dependent mediation of ATP release from HMC-1 cells by 1 nM–10 μM exo-5-HT). ### $p < 0.001$ vs. baseline (absent of exo-5-HT). (B,C) Effects of 5-HT receptors inhibition on exo-5-HT induced ATP release from HMC-1 cells. Meth. (100 nM) or Gran. (100 nM) was introduced to cells 10 min before 5-HT intervention and was present during the entire process. ** $p < 0.01$. (D) Response of 5-HT release from HMC-1 cells to 10 nM–100 μM exogenous ATP intervention. (E) WB determination of 5-HT$_{1A, B, D, F}$ subtypes identification on HMC-1 cells. These are representative blots from n = 4–6 recordings. (F) Effects of 5-HT$_{1A}$R inhibition on exo-5-HT induced ATP release from HMC-1 cells. WAY-100635 (WAY., 100 nM) was introduced to cells 10 min before exo-5-HT intervention and was present during the entire process. * $p < 0.05$ and *** $p < 0.001$. (G,H) Effect of 5-HT$_{1A}$ receptor-inhibition on AP-relieving tactile allodynia and thermal hyperalgesia of the injured plantar, respectively. WAY. (2 mg/mL, 20 μL) was pre-injected at ST 36. Data illustrate the final behavioral tests. ** and ***, $p < 0.01$ and 0.001, respectively. Numbers above each column/circle denote sample size (n). In (F), *Unpaired t-test* was performed. In the rest panels, *one-way ANOVA* and *LSD. test* were used.

Pretreatment HMC-1 with Methiothepin (100 nM) significantly impaired ATP release induced by 1 μM- and 10 μM-exo-5-HT as well. In contract, Granisetron (100 nM) had null effect (Fig. 2B,C). Similar results were also obtained from rat MCs, RBL2H3 cell line (**Supplementary Fig. 4**). However, exogenous ATP had null effect on 5-HT release (Fig. 2D). These results suggest that 5-HT mediates ATP release from MCs via activating 5-HTRs, especially 5-HT$_1$Rs.

5-HT$_1$Rs family comprises A, B, D, E and F subtypes. Currently, all subtypes except for E have been reported to be involved in transducing of peripheral pain [28]. Among them, subtype A has been repeatedly confirmed to be expressed in human and rat MCs. Our WB determination revealed that 5-HT$_{1A}$R markedly expressed in HMC-1 cells (Fig. 2E). Subsequently, we examined the effect of WAY-100635 (WAY.), a specific antagonist of 5-HT$_{1A}$R. The result uncovered that pretreatment HMC-1 with WAY. (100 nM) significantly impaired ATP release induced by 1 μM (n = 5–6, $p = 2.8 \times 10^{-6}$ vs. -WAY.) and 10 μM (n = 5, $p = 0.0020$ vs. -WAY.) exo-5-HT (Fig. 2F). Taken together, these results indicate that 5-HT mediates ATP release from MCs via activating 5-HT$_{1A}$R subtype.

Based on these findings, we returned to the test *in vivo*. Behavioral tests found that pre-injection of WAY. (2 mg/mL, 20 μL) in ST 36 canceled AP analgesic effect on PWT (n = 5–6, $p = 8.1 \times 10^{-5}$ vs. AP) and PWL (n = 4–6, $p = 0.0003$ vs. AP) (Fig. 2G,H). Taken these results

together, we infer that contribution of MC-associated 5-HT release to AP analgesia mechanism might be due to mediate ATP release from MCs or other adjacent cells by activating 5-HT_{1A}R.

3.3 Needling-Induced Inters. ATP Mobilization in Acupoint was Involved in AP Analgesia Possibly via Producing Adenosine or Propagating AP Signals

AP-induced adenosine accumulation and activation of local adenosine A_1Rs contribute to initiate analgesia [14]. Such adenosine mobilization might partially rely on the hydrolyzation of ATP. Our microdialysate determination revealed that both adenosine and ATP in the interstitial space of treated acupoint were significantly potentiated by needling (adenosine, from baseline of 58.4 nM ± 6.9 nM to 76.8 nM ± 9.1 nM, n = 9, p = 0.0250; ATP, from baseline of 3.4 nM ± 1.4 nM to 56.0 nM ± 12.5 nM, n = 4, p = 0.0269). More importantly, the presence of ARL (100 μM, 50 μL), a non-specific inhibitor of ecto-ATPase, in acupoint further potentiated AP-induced ATP accumulation (136.3 nM ± 22.7 nM, n = 4, p = 0.0258 vs. AP group) (Fig. 3A), indicating the activities of ecto-ATPase and hydrolyzation of inters. ATP existed in the treated acupoint. Further behavioral tests demonstrated that AP analgesic effect was impaired by ARL (100 μM, 50 μL) (n = 4, p = 0.0233), and was duplicated by CCPA (0.04 mg/mL, 20 μL), an agonist of adenosine A1Rs (n = 5, p = 0.9773) (Fig. 3B). Taken together, these findings suggest that mobilization of inters. ATP via primary or secondary release contributes to antinociceptive mechanism of AP by promoting adenosine production.

Calcium wave propagation (CWP) is supposed as the basis of ATP regeneration, which facilitates the localized propagation of biological signals [29]. CWP has been uncovered in mouse treated acupoint [30]. Our previous work had found a similar CWP and cascaded ATP release in HMC-1 cells when perturbed by hypotonic shock, in which primary mechanosensitive ATP released induced secondary ATP release via activating $P2Y_{13}$ or $P2X_7$ receptors [15].

The present work found an additional 5-HT-induced secondary ATP release. We hypothesized that besides as the precursor of adenosine, such cascaded ATP release could amply and propagate the localized needling signals. Our WB determination revealed that some components for forming CWP/ATP regeneration, including Panx-1, connexin 43 and $P2Y_1$ were expressed in HMC-1 cells (**Supplementary Fig. 5**). Subsequently, further behavioral tests observed that inhibition of local purinergic receptors (P2 receptors) by Suramin (100 μM, 50 μL) (Relative PWT: reduced from 0.9 ± 0.0 to 0.7 ± 0.1, n = 4, p = 0.0057 vs. AP and Relative PWL: reduced from 0.9 ± 0.0 to 0.6 ± 0.0, n = 4, p = 0.0269 vs. AP) or PPADS (100 μM, 50 μL) (Relative PWT: reduced from 0.9 ± 0.0 to 0.7 ± 0.1, n = 4, p = 0.0009 vs. AP and Relative PWL: reduced from 0.9 ± 0.0 to 0.8 ± 0.1, n = 4, p = 0.0749 vs. AP) partially suppressed the AP-relieved tactile allodynia and thermal hyperalgesia (Fig. 3C,D). This finding implies that besides adenosine and A1Rs, ATP/ADP and related P2 receptors in acupoint also play a role to mediate AP analgesia and its underlying mechanism might be the ATP-related CWP.

4. Discussion

4.1 Local MC-Associated 5-HT Reflected the State of Acupoints

5-HT-immunopositive MCs are present in acupoints and become more when the acupoints are sensitized [8,9]. Acupoint sensitization means acupoints transformed dynamically from a “silent” state to an “activated” one when the body is suffering from some corresponding diseases [31]. Neurogenic inflammation is one of the biological bases for acupoint sensitization due to dorsal root reflex or axon reflex [32,33]. In the present work, we noticed that compared to normal rats, ST 36 in model group had higher inters. 5-HT level at baseline (**Supplementary Fig. 1**). Here, the onset site and treated acupoint are innovated by tibial nerve and peroneal nerve, respectively, branches of sciatic nerve, suggesting that they are innovated by the same or adjacent peripheral ganglia and spinal segments. Similarly, in knee osteoarthritis rats, 5-HT together with tryptase and histamine are upregulated in the nearby acupoints, Yanglingquan acupoint and Heding acupoint rather than Weizhong acupoint [34]. The greatest benefit of acupoint sensitization is to facilitate AP to exert better therapeutic effect. Our previous work had revealed that AP modulated higher ATP accumulated in AA rats than in normal ones [19]. Systematic review demonstrates the comparative superiority of needle stimulation of sensitized points over non-sensitized points, especially for some pain syndromes [31].

Degranulation of local subcutaneous MCs induced by needling has been often reported [1]. While the certain associated mediators are not well quantitatively studied. Beside adenosine assessed in our previous work [4], the current study clearly recorded MC-associated 5-HT mobilization (Fig. 1A,B). Based on the microdialysis recovery percentage [19] and considering dilution by diffusion and/or convection into the bulk extracellular volume [23], the real concentration of inters. 5-HT at the cell surface was more than 500 ng/mL (2.8 μM). This concentration is within the range we used in our test *in vitro* (1 and 10 μM), which is sufficient to activate 5-HT_{1A}R (Fig. 2F). Additionally, this concentration range can activate 5-HT_{1A}R [24], 5-HT_{2A}R [17] and 5-HT_3R [25], implying various functions are involved, for example, influence the differentiation, proliferation, migration, adherence and life-span of skin cells, regulate the tonus of blood vessels and influence nerve transmission [35]. The potentiated inters. 5-HT could last for more than 2.5 h (Fig. 1A,B). It might contribute to the maintenance of AP effect.

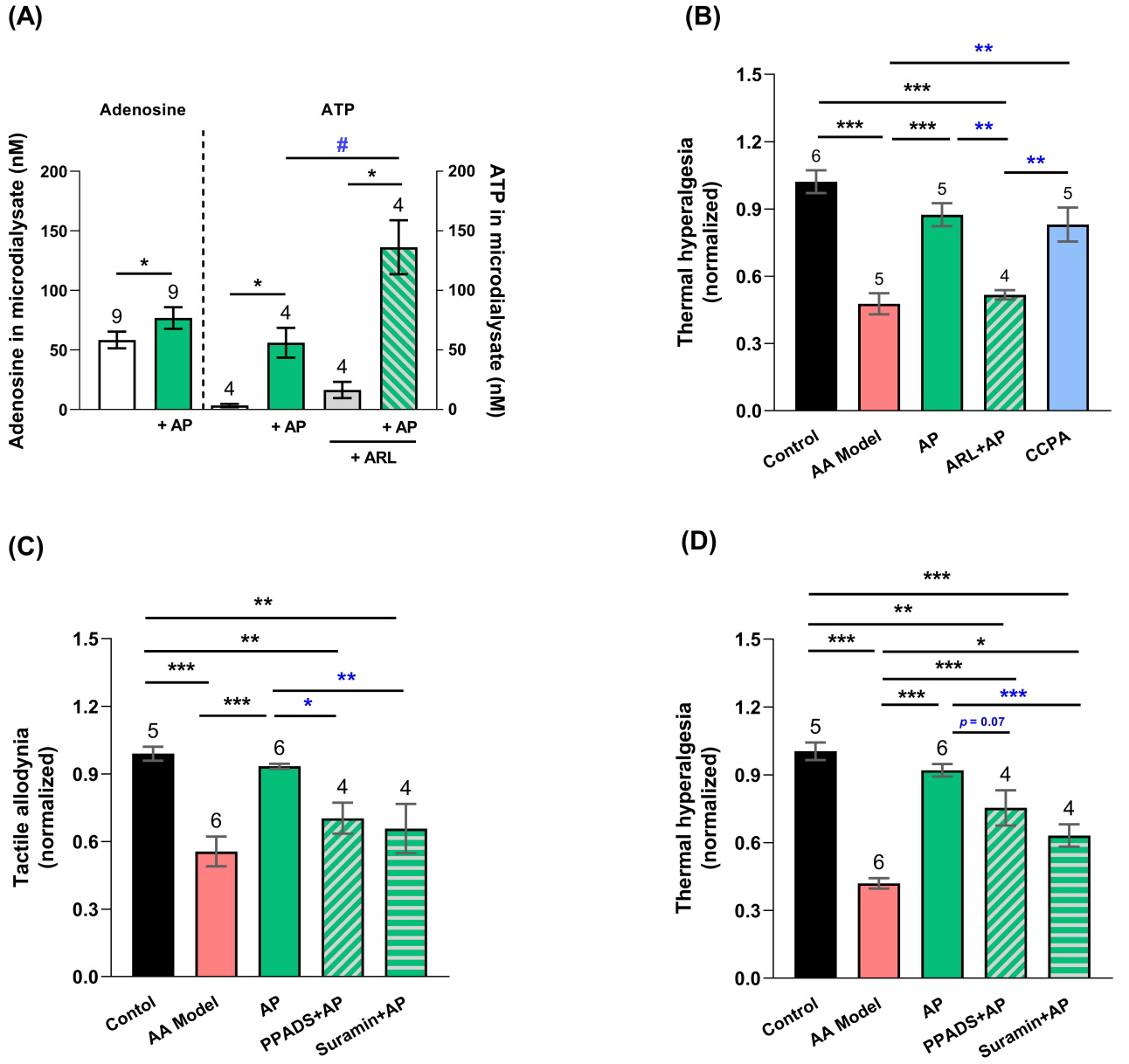

Fig. 3. AP-induced the mobilization of adenosine (Ado) and inters. ATP in the treated acupoint contributed to the anti-nociceptive effect of AP (n = 4–9, mean ± SE). (A) Changes of Adenosine and ATP levels in the interstitial space in ST 36 of AA model rats before (-AP) and after (+AP) needling. Adenosine was determined with HPLC. ATP was assayed with L-L method. ARL (100 μM, 50 μL) was pre-injected at ST 36 20 min prior to AP. * $p < 0.05$ by paired *t-test*. # $p < 0.05$ by unpaired *t-test*. (B) Involvement of inters. ATP hydrolyzation and Adenosine A1 receptors in AP-analgesic effect. ARL (100 μM, 50 μL) or CCPA (0.04 mg/mL, 20 μL) was pre-injected at ST 36. ** and ***, $p < 0.01$ and 0.001, respectively. (C,D) Effects of P2 receptor-inhibition on AP-analgesia in tactile allodynia and thermal hyperalgesia, respectively. *, ** and ***, $p < 0.05$, 0.01 and 0.001, respectively. In (B–D), data illustrate the final behavioral tests and *one-way ANOVA* and *LSD. test* were used. Numbers above each column denote sample size (n).

4.2. 5-HT Induced ATP Release by Activating 5-HT$_{1A}$R

The activation of MCs can easily establish a positive feedback loop. Matrix metalloproteinase-9 [36] or ATP [15] secreted from MCs, in turn, activate MCs. In the present work, unidirectional regulation of 5-HT on ATP release via activation of 5-HT$_{1A}$R was another example of this self-activation mechanism (Fig. 2A). Other cells can also be activated, as long as they express 5-HT$_{1A}$R, for example, melanocytes [37]. Similar finding has been reported in type II cells of rat's carotid body, in which exo-5-HT increases $[Ca^{2+}]_i$ partially via activating Panx-1 channels, implying 5-HT might facilitate ATP release [17]. Although we demonstrated that MCs express Panx-1 channels (**Supplementary Fig. 5**), whether they assisted ATP re-

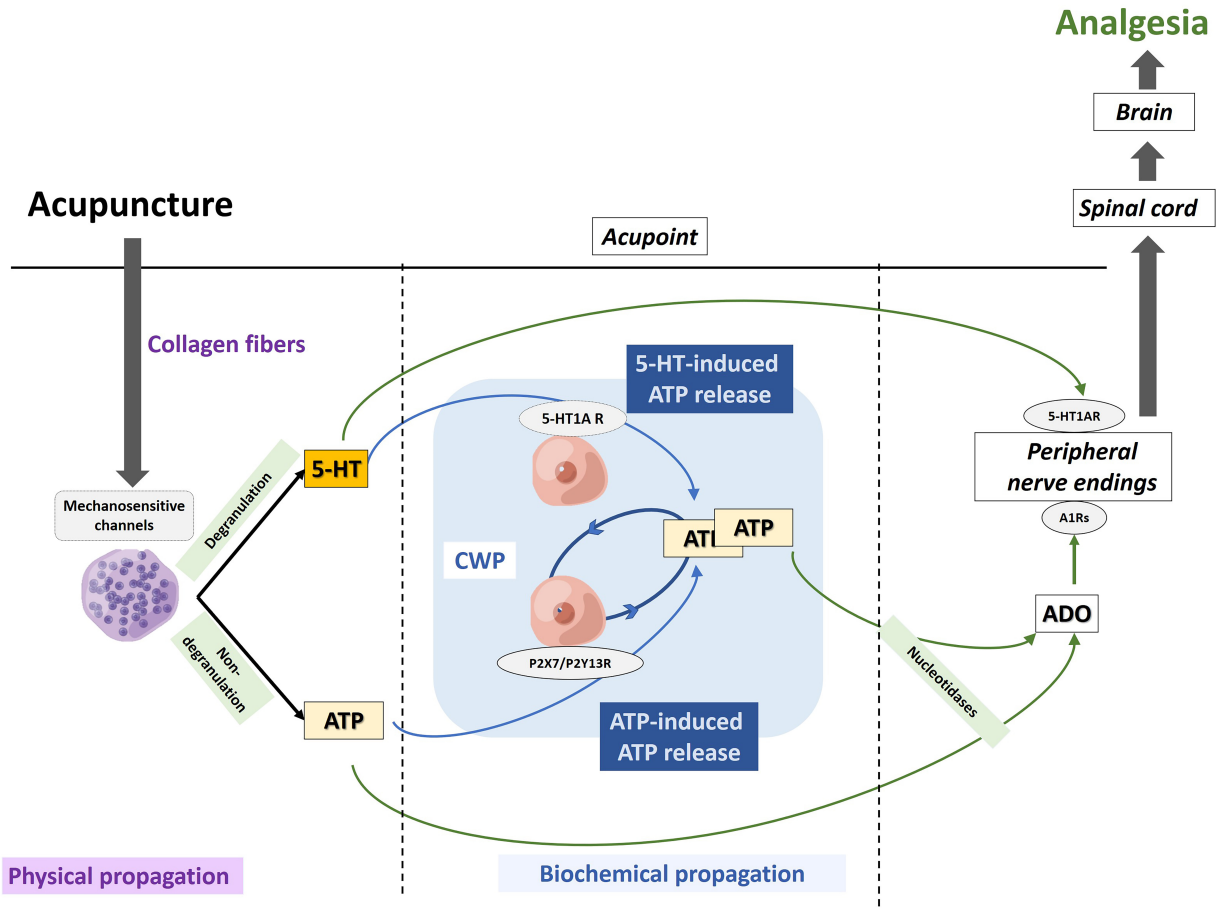

Fig. 4. Schematic representation of how MC-associated 5-HT in acupoints participates in AP analgesia. Collagen fibers winding within acupoints generated by AP activates MCs to release 5-HT and ATP in the manner of degranulation and non-degranulation, respectively. 5-HT facilitates ATP release by activating 5-HT_{1A}Rs in the adjacent cells. The released ATP hydrolyzes into Adenosine with the aid of ecto-nucleotidases, which contributes to AP analgesia through acting A1 receptors on the local nerve endings; or activates P2 receptors in the adjacent cells to forming CWP via opening ATP channels to amplify AP signals. Eventually, these signals are sensed by peripheral sensory ganglia and further ascend to central nerve system.

lease still need further determination.

Additionally, interaction between 5-HT mediated signaling and ATP-related P2X receptors have been characterized. While it is mainly inhibitory rather than facilitative. In submucosal neurons of guinea pigs, whole-cell recordings reveal that 5-HT_3Rs and P2X channels negatively modulate each other when they are simultaneously activated [38]. 5-HT_{3A}R and P2X2 channels were found to co-immunoprecipitate constitutively and be associated in clusters in cultured myenteric neurons of guinea pig as well as expression cells [39]. 5-HT_3Rs in pelvic afferent neurons is up-regulated in mice lacking P2X2 or P2X3 receptor genes [25].

4.3. Inters. ATP Contributed to AP-Analgesia as the Precursor of Adenosine

We demonstrated an upregulation of inters. ATP in the treated acupoint by needling (Fig. 3A), and prevention its hydrolysis by ARL impaired AP-analgesic effect (Fig. 3B). These findings imply that ATP mobilization and subsequent hydrolysis are vital steps towards antinociception during needling. The aggregated inters. ATP originates from cell lysis and non-lytic release as well. Regarding the former, needle piercing and subsequent manipulation can cause tissue damage. As reported, damage-associated molecules, high mobility group box 1 protein (HMGB1) and toll-like receptor 4 (TLR4), increases in the treated acupoint of normal rats by 2-min needling [40]. The non-lytic release of ATP results from the primary ATP secretion via activating mechanosensitive channels [1] and secondary ATP release induced by ATP or 5-HT, as we discussed above. But unlike 5-HT, mechanosensitive ATP release via non-degranulation approach (**Supplementary Fig. 2A**).

Adenosine, as the downstream product of ATP, aggregates in acupoints too [14,41]. The current work and other similar study [14] observed that pharmacologic activation of adenosine A1R in acupoints had antinociceptive effect (Fig. 3B), in which A1R are supposed to be situated on lo-

cal nerve terminals [14]. Like ATP, adenosine might also release out via lytic and non-lytic approaches [42].

We inferred the presence of ecto-ATPases in the acupoint based on the reinforcing effect of ARL on inters. ATP accumulation (Fig. 3A). NTPDases family is the major nucleotide-hydrolyzing enzymes, which degrade ATP to AMP with intermediate formation of ADP [43]. Our previous work revelated that rats ST 36 acupoint expressed mRNA of NTPDase-1, -2,-3 [16]. Combined with ecto-AMPase, for example, ecto-5'-nucleotidase (Nt5e), can fulfill the production of adenosine. In the skin, Langerhans cells express NTPDase-1 [44], keratinocytes express NTPDase-3 [45] and Nt5e [46]. Behavioral evidences in mice show that nucleotidases prostatic acid phosphatase (PAP) [47] and Nt5e [48,49] have anti-nociceptive effects at spinal cord level via promoting adenosine production. Injection of PAP into Weizhong acupoint (BL 40) has analgesic effect similar to AP treatment [50].

4.4 Inters. ATP may Facilite Localized Propagation of Needling Signals

Needling manipulations generate mechanical stimulation that is transmitted to the wider and deeper space by subcutaneous collagen fibers twisting around the needles [51,52]. This is the "physical propagation" of needling signals [3]. In addition, "biochemical propagation" also occurs. As reported, AP-generated acoustic shear waves can cause $[Ca^{2+}]_i$ shock and CWP *in vitro* and *in vivo* [30]. CWP permits regenerative signal amplification. It has been proposed that non-neural cells respond to mechanical stimulation by ATP release and send signals to nerve terminals via CWP [30]. CWP formation is commonly based on ATP regeneration [29]. Compelling evidence indicates that ATP-mediated ATP release involves P2YRs [53], among which P2Y1R is the most documented [54,55]. Our previous work had observed a similar CWP phenomenon in cultured MCs in response to mechanical stimulation, which relied on the activation of $P2Y_{13}$ and $P2X_7$ receptors via "ATP-induced ATP release" mechanism [15]. 5-HT-mediated ATP release uncovered in this work is a good complement to ATP regeneration, then facilitate CWP formation. The necessary components for CWP, including P2Y1 receptors, Connexin 43 and Panx-1 channels are expressed in mice acupoint (ST 36) [20]. In the present work, WB determined their presence in MCs (**Supplementary Fig. 5**). Furthermore, Panx-1 [56] and connexin 43 [57] are been reported to express in skeletal muscle and fibroblasts that are also main tissues contained in acupoints. Hence, besides as the precursor of adenosine, inters. ATP in acupoint might exert function of transmitting the needling signals. This hypothesis was partially confirmed by the inhibitory effect of Suramin and PPADS on ATP regeneration in MCs in our previous work [15] and AP-analgesic effect in the present study (Fig. 3C,D).

4.5 Contribution of 5-HT to AP Analgesia is Might via Direct Activating 5-HT_{1A}R

We found that 5-HTRs, especially 5-HT_{1A}R, in acupoint played role in the initiation mechanism of AP-analgesia (Fig. 1E,F and Fig. 2G,H). Beside facilitating needling signals as we discussed above, 5-HT might contribute to AP analgesic effect by activating 5-HT_{1A}R on the adjacent peripheral nerve endings. As we investigated previously, AP-activated MCs were eventually involved in the analgesic effect via transmitting the biological signals to the nearby nerve endings, then ascending to central nerve system [58]. 5-HT_1Rs class represents the most complex subtype families of 5-HTRs, which comprise of five receptor subtypes, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}. Although 5-HT_{1A}R activation is reported to elicit hyperalgesia [59,60], later researches reveal its anti-nociceptive effect. Peripheral sensory nerve endings are the main location of 5-HT_{1A} subtype [61]. Administered intrathecally with (±)-8-hydroxydipropylaminotetralin hydrobromide (8-OH-DPAT), a 5-HT_{1A}R agonist, was to suppressed the second phase of formalin-induced aversive responses in the 5,7-dihydroxytryptamine treated rats [62]. Another research using 8-OH-DPAT also suggested an analgesic action is exerted by 5-HT_{1A}R in the formalin model of tonic nociceptive pain in rats [63]. It still needs further work to explore whether 5-HT mediates AP analgesia by activating 5-HT_{1A}R situated on nearby nerve endings.

5. Conclusions

The present study supports the critical role of MC-associated 5-HT in the initiation mechanism of AP analgesia. The underlying mechanisms involve 5-HT_{1A}R-mediated ATP release and its downstream events, including adenosine generation and CWP formation. Fig. 4 demonstrates our conclusion in detail.

Abbreviations

5-HT, 5-hydroxytryptamine, serotonin; 5-HTRs, 5-HT receptors; 5-HT_1Rs, 5-HT1 receptors; 5-HT_{1A}Rs, 5-HT1A receptors; 5-HT_3Rs, 5-HT3 receptors; AA, adjuvant arthritis; AP, acupuncture; ARL67156, 6-N,N-Diethyl-β-γ-dibromomethylene-D-adenosine-5'-triphosphate; ATP, adenosine triphosphate; $[Ca^{2+}]$i, intracellular calcium concentration; CCPA, 2-Chloro-N6-cyclopentyladenosine; CFA, complete Freund's adjuvant; CRO, sodium cromolyn; CWP, calcium wave propagation; DMSO, dimethyl sulfoxide; Inters. 5-HT, interstitial 5-HT; Inters. ATP, interstitial ATP; ELISA, enzyme linked immunosorbent assay; Exo-5-HT, exogenous 5-HT; HBSS, Hank's balanced salt solution; HPLC, High-performance liquid chromatography; MCs, mast cells; Panx-1, pannexin 1; PPADS, pyridoxal phosphate-6-azo tetrasodium salt hydrate; P2 receptors, purinergic P2 receptors; PWL, paw withdraw latency; PWT, paw withdraw threshold; WAY-100635, N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-

N-2-pyridinylcyclohexanecarboxamide maleate salt.

Availability of Data and Materials

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

Author Contributions

These should be presented as follows: L-NW, X-YS, G-HD and DZ designed the research study. B-RL, Y-JL and J-WX performed the research. B-RL and Y-JL performed the data analysis and interpretation. MH provided help and advice on methodology. B-RL and L-NW wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by The Animal Experiment Committee of the Animal Experimental Center of Shanghai University of Traditional Chinese Medicine (Animal Ethics No.: PZSHUTCM200911014; date of approval: Sep. 11th, 2020).

Acknowledgment

We are grateful to all members of our research groups at Shanghai Key Laboratory of Acupuncture Mechanism and Acupoint Function of Fudan University, at Acupuncture and Moxibustion College of Shanghai University of Traditional Chinese Medicine, and at Shanghai Research Centre for Acupuncture and Meridians, in particular to Xuezhi Wang, Ph.D., Yong Wu, Ph.D., Yi Yu, Ph.D., Mingzhu Sun and Yingchen Li for their suggesstions about hehaviroal tests, and WeiminZuo, M.D. for her advices about microdialysis technique.

Funding

This research was funded by National Natural Science Foundation of China, grant number 81574076, 82174488, 81590953, 81574053; Budget Research Project of Shanghai Education Commission, grant number 2021LK098; Shanghai Key Laboratory for Acupuncture Mechanism and Acupoint Function, grant number 21DZ2271800; Shanghai Natural Science Foundation, Grant Number 22ZR1461500.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2801001.

References

[1] Wang LN, Wang XZ, Li YJ, Li BR, Huang M, Wang XY, *et al.* Activation of Subcutaneous Mast Cells in Acupuncture Points Triggers Analgesia. Cells. 2022; 11: 809.

[2] Zhang D, Ding G, Shen X, Yao W, Zhang Z, Zhang Y, *et al.* Role of mast cells in acupuncture effect: a pilot study. Explore. 2008; 4: 170–177.

[3] Ding G, Zhang D, Huang M, Wang L, Yao W. The function of collagen and mast cells at acupoints. Springer: New York. 2012.

[4] Huang M, Wang X, Xing B, Yang H, Sa Z, Zhang D, *et al*. Critical roles of TRPV2 channels, histamine H1 and adenosine a1 receptors in the initiation of acupoint signals for acupuncture analgesia. Scientific Reports. 2018; 8: 6523.

[5] Wang L, Sikora J, Hu L, Shen X, Grygorczyk R, Schwarz W. ATP Release from Mast Cells by Physical Stimulation: a Putative Early Step in Activation of Acupuncture Points. Evidence-Based Complementary and Alternative Medicine. 2013; 2013: 1–7.

[6] He W, Wu M, Jing XH, Bai W, Zhu B, Yu X. Entity of acupoint: kinetic changes of acupoints in histocytochemistry. Zhongguo Zhen Jiu. 2015; 35: 1181–1186. (In Chinese)

[7] Wingren U, Wasteson A, Enerbäck L. Storage and Turnover of Histamine, 5-Hydroxytryptamine and Heparin in Rat Peritoneal Mast Cells *in vivo*. International Archives of Allergy and Immunology. 1983; 70: 193–199.

[8] Wu M, Xu D, Bai W, Cui J, Shu H, He W, *et al*. Local cutaneous nerve terminal and mast cell responses to manual acupuncture in acupoint LI4 area of the rats. Journal of Chemical Neuroanatomy. 2015; 68: 14–21.

[9] Dimitrov N, Atanasova D, Tomov N, Sivrev D, Lazarov N. Acupuncture causes serotonin release by mast cells. Romanian Journal of Morphology and Embryology. 2017; 58: 961–968.

[10] Sommer C. Serotonin in pain and analgesia: actions in the periphery. Molecular Neurobiology. 2004; 30: 117–125.

[11] Sandoval-Talamantes AK, Gómez-González BA, Uriarte-Mayorga DF, Martínez-Guzman MA, Wheber-Hidalgo KA, Alvarado-Navarro A. Neurotransmitters, neuropeptides and their receptors interact with immune response in healthy and psoriatic skin. Neuropeptides. 2020; 79: 102004.

[12] Domocos D, Selescu T, Ceafalan LC, Iodi Carstens M, Carstens E, Babes A. Role of 5-HT1A and 5-HT3 receptors in serotonergic activation of sensory neurons in relation to itch and pain behavior in the rat. Journal of Neuroscience Research. 2020; 98: 1999–2017.

[13] Burnstock G. Purinergic Mechanisms and Pain. Pharmacological Mechanisms and the Modulation of Pain. 2016; 75: 91–137.

[14] Goldman N, Chen M, Fujita T, Xu Q, Peng W, Liu W, *et al*. Adenosine a1 receptors mediate local anti-nociceptive effects of acupuncture. Nature Neuroscience. 2010; 13: 883–888.

[15] Shen D, Shen X, Schwarz W, Grygorczyk R, Wang L. $P2Y_{13}$ and $P2X_7$ receptors modulate mechanically induced adenosine triphosphate release from mast cells. Experimental Dermatology. 2020; 29: 499–508.

[16] Zheng Y, Zuo W, Shen D, Cui K, Huang M, Zhang D, *et al.* Mechanosensitive TRPV4 Channel-Induced Extracellular ATP Accumulation at the Acupoint Mediates Acupuncture Analgesia of Ankle Arthritis in Rats. Life. 2021; 11: 513.

[17] Murali S, Zhang M, Nurse CA. Evidence that 5-HT stimulates intracellular Ca(2+) signalling and activates pannexin-1 currents in type II cells of the rat carotid body. The Journal of Physiology. 2017; 595: 4261–4277.

[18] Shen D, Zheng Y, Zhang D, Shen X, Wang L. Acupuncture modulates extracellular ATP levels in peripheral sensory nervous system during analgesia of ankle arthritis in rats. Purinergic Signalling. 2021; 17: 411–424.

[19] Zuo WM, Li YJ, Cui KY, Shen D, Zhang D, Zheng YW, *et al*. The real-time detection of acupuncture-induced extracellular ATP mobilization in acupoints and exploration of its role in acupuncture analgesia. Purinergic Signalling. 2022. (online ahead of print)
[20] Wu S, Chen W, Hsieh C, Lin Y. Abundant expression and functional participation of TRPV1 at Zusanli acupoint (ST36) in mice: mechanosensitive TRPV1 as an "acupuncture-responding channel". BMC Complementary and Alternative Medicine. 2014; 14: 96.
[21] Mikolajewicz N, Mohammed A, Morris M, Komarova SV. Mechanically stimulated ATP release from mammalian cells: systematic review and meta-analysis. Journal of Cell Science. 2018; 131: jcs223354.
[22] Zhang D, Spielmann A, Wang L, Ding G, Huang f, Gu Q, *et al*. Mast-Cell Degranulation Induced by Physical Stimuli Involves the Activation of Transient-Receptor-Potential Channel TRPV2. Physiological Research. 2012; 61: 113–124.
[23] Joseph SM, Buchakjian MR, Dubyak GR. Colocalization of ATP Release Sites and Ecto-ATPase Activity at the Extracellular Surface of Human Astrocytes. Journal of Biological Chemistry. 2003; 278: 23331–23342.
[24] Subhash MN, Srinivas BN, Vinod KY, Jagadeesh S. Modulation of 5-HT 1a receptor mediated response by fluoxetine in rat brain. Journal of Neural Transmission. 2000; 107: 377–387.
[25] Ma B, Wynn G, Dunn PM, Burnstock G. Increased 5-HT3-mediated signalling in pelvic afferent neurons from mice deficient in P2X2 and/or P2X3 receptor subunits. Purinergic Signalling. 2006; 2: 481–489.
[26] Schwartz LB, Metcalfe DD, Miller JS, Earl H, Sullivan T. Tryptase Levels as an Indicator of Mast-Cell Activation in Systemic Anaphylaxis and Mastocytosis. New England Journal of Medicine. 1987; 316: 1622–1626.
[27] Dvorak AM. Basophil and Mast Cell Degranulation and Recovery. Plenum Press: New York. 1991.
[28] Granados-Soto V, Argüelles CF, Rocha-González HI, Godínez-Chaparro B, Flores-Murrieta FJ, Villalón CM. The role of peripheral 5-HT1a, 5-HT1B, 5-HT1D, 5-HT1E and 5-HT1F serotonergic receptors in the reduction of nociception in rats. Neuroscience. 2010; 165: 561–568.
[29] Ceriani F, Pozzan T, Mammano F. Critical role of ATP-induced ATP release for Ca2+ signaling in nonsensory cell networks of the developing cochlea. Proceedings of the National Academy of Sciences of the United States of America. 2016; 113: E7194–E7201.
[30] Li G, Liang J, Li P, Yao X, Pei PZ, Li W, *et al*. Physiology and cell biology of acupuncture observed in calcium signaling activated by acoustic shear wave. PflüGers Archiv - European Journal of Physiology. 2011; 462: 587–597.
[31] Tan H, Tumilty S, Chapple C, Liu L, McDonough S, Yin H, *et al*. Understanding Acupoint Sensitization: a Narrative Review on Phenomena, Potential Mechanism, and Clinical Application. Evidence-Based Complementary and Alternative Medicine. 2019; 2019: 6064358.
[32] Kim D, Ryu Y, Hahm DH, Sohn BY, Shim I, Kwon OS, *et al*. Acupuncture points can be identified as cutaneous neurogenic inflammatory spots. Scientific Reports. 2017; 7: 15214.
[33] Zhang M, Guo H, Ma Y, Xu F, Bai F, Liang S, *et al.* Acupoint sensitization is associated with increased excitability and hyperpolarization-activated current (I_h) in C- but not Adelta-type neurons. Neuroscience. 2019; 404: 499–509.
[34] Ding N, Jiang J, Qin P, Wang Q, Hu J, Li Z. Mast cells are important regulator of acupoint sensitization via the secretion of tryptase, 5-hydroxytryptamine, and histamine. PLoS ONE. 2018; 13: e0194022.
[35] Nordlind K, Azmitia EC, Slominski A. The skin as a mirror of the soul: exploring the possible roles of serotonin. Experimental Dermatology. 2008; 17: 301–311.
[36] Xu L, Cai Z, Yang F, Chen M. Activation-induced upregulation of MMP9 in mast cells is a positive feedback mediator for mast cell activation. Molecular Medicine Reports. 2017; 15: 1759–1764.
[37] Lundeberg L, El-Nour H, Mohabbati S, Morales M, Azmitia E, Nordlind K. Expression of serotonin receptors in allergic contact eczematous human skin. Archives of Dermatological Research. 2002; 294: 393–398.
[38] Barajas-López C, Montaño LM, Espinosa-Luna R. Inhibitory interactions between 5-HT3 and P2X channels in submucosal neurons. American Journal of Physiology: Gastrointestinal and Liver Physiology. 2002; 283: G1238–G1248.
[39] Boué-Grabot E, Barajas-López C, Chakfe Y, Blais D, Bélanger D, Émerit MB, *et al*. Intracellular Cross Talk and Physical Interaction between Two Classes of Neurotransmitter-Gated Channels. The Journal of Neuroscience. 2003; 23: 1246–1253.
[40] Qiang XI, Cui R, Jin G, Guo YM. Preliminary research on the role of TLR4 at acupoints in the inflammatory reaction produced by acupuncture. Tianjin Journal of Traditional Chinese Medicine. 2015; 32: 88–92.
[41] Takano T, Chen X, Luo F, Fujita T, Ren Z, Goldman N, *et al*. Traditional Acupuncture Triggers a Local Increase in Adenosine in Human Subjects. The Journal of Pain. 2012; 13: 1215–1223.
[42] Marquardt DL, Gruber HE, Wasserman SI. Adenosine release from stimulated mast cells. Proceedings of the National Academy of Sciences. 1984; 81: 6192–6196.
[43] Zimmermann H, Zebisch M, Sträter N. Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signalling. 2012; 8: 437–502.
[44] Mizumoto N, Kumamoto T, Robson SC, Sévigny J, Matsue H, Enjyoji K, *et al*. CD39 is the dominant Langerhans cell–associated ecto-NTPDase: Modulatory roles in inflammation and immune responsiveness. Nature Medicine. 2002; 8: 358–365.
[45] Vongtau HO, Lavoie EG, Sévigny J, Molliver DC. Distribution of ecto-nucleotidases in mouse sensory circuits suggests roles for nucleoside triphosphate diphosphohydrolase-3 in nociception and mechanoreception. Neuroscience. 2011; 193: 387–398.
[46] Ogawa Y, Kinoshita M, Mizumura N, Miyazaki S, Aoki R, Momosawa A, *et al*. Purinergic Molecules in the Epidermis. Journal of Investigative Dermatology. 2018; 138: 2486–2488.
[47] Zylka MJ, Sowa NA, Taylor-Blake B, Twomey MA, Herrala A, Voikar V, *et al*. Prostatic Acid Phosphatase is an Ectonucleotidase and Suppresses Pain by Generating Adenosine. Neuron. 2008; 60: 111–122.
[48] Sowa NA, Taylor-Blake B, Zylka MJ. Ecto-5'-Nucleotidase (CD73) Inhibits Nociception by Hydrolyzing AMP to Adenosine in Nociceptive Circuits. Journal of Neuroscience. 2010; 30: 2235–2244.
[49] Sowa NA, Voss MK, Zylka MJ. Recombinant ecto-5'-nucleotidase (CD73) has long lasting antinociceptive effects that are dependent on adenosine A1 receptor activation. Molecular Pain. 2010; 6: 20.
[50] Hurt JK, Zylka MJ. PAPupuncture has Localized and Long-Lasting Antinociceptive Effects in Mouse Models of Acute and Chronic Pain. Molecular Pain. 2012; 8: 28.
[51] Yu X, Ding G, Huang H, Lin J, Yao W, Zhan R. Role of Collagen Fibers in Acupuncture Analgesia Therapy on Rats. Connective Tissue Research. 2009; 50: 110–120.
[52] Langevin HM, Churchill DL, Wu J, Badger GJ, Yandow JA, Fox JR, *et al*. Evidence of Connective Tissue Involvement in Acupuncture. The FASEB Journal. 2002; 16: 872–874.
[53] Zimmermann H. Extracellular ATP and other nucleotides—ubiquitous triggers of intercellular messenger release. Purinergic

Signalling. 2016; 12: 25–57.
[54] Weng J, Hsu T, Sun SH. Functional characterization of P2Y1 versus P2X receptors in RBA-2 astrocytes: Elucidate the roles of ATP release and protein kinase C. Journal of Cellular Biochemistry. 2008; 104: 554–567.
[55] Svobodova I, Bhattaracharya A, Ivetic M, Bendova Z, Zemkova H. Circadian ATP Release in Organotypic Cultures of the Rat Suprachiasmatic Nucleus Is Dependent on P2X7 and P2Y Receptors. Frontiers in Pharmacology. 2018; 9: 192.
[56] Buvinic S, Almarza G, Bustamante M, Casas M, López J, Riquelme M, *et al*. ATP Released by Electrical Stimuli Elicits Calcium Transients and Gene Expression in Skeletal Muscle. Journal of Biological Chemistry. 2009; 284: 34490–34505.
[57] Pistorio AL, Ehrlich HP. Modulatory effects of connexin-43 expression on gap junction intercellular communications with mast cells and fibroblasts. Journal of Cellular Biochemistry. 2011; 112: 1441–1449.
[58] Huang H, Zhan R, Yu XJ, Zhang D, Li WM, Ding GH. Effects of acupoint-nerve block on mast cell activity, manual acupuncture- and electroacupuncture-induced analgesia in adjuvant arthritis rats. Zhen Ci Yan Jiu. 2009; 34: 31–35, 56. (In Chinese)
[59] Taiwo YO, Levine JD. Serotonin is a directly-acting hyperalgesic agent in the rat. Neuroscience. 1992; 48: 485–490.
[60] Millan MJ. Serotonin and pain: evidence that activation of 5-HT1A receptors does not elicit antinociception against noxious thermal, mechanical and chemical stimuli in mice. Pain. 1994; 58: 45–61.
[61] Barnes NM, Ahern GP, Becamel C, Bockaert J, Camilleri M, Chaumont-Dubel S, *et al*. International Union of Basic and Clinical Pharmacology. CX. Classification of Receptors for 5-hydroxytryptamine; Pharmacology and Function. Pharmacological Reviews. 2021; 73: 310–520.
[62] Oyama T, Ueda M, Kuraishi Y, Akaike A, Satoh M. Dual effect of serotonin on formalin-induced nociception in the rat spinal cord. Neuroscience Research. 1996; 25: 129–135.
[63] Bardin L, Tarayre JP, Koek W, Colpaert FC. In the formalin model of tonic nociceptive pain, 8-OH-DPAT produces 5-HT1A receptor-mediated, behaviorally specific analgesia. European Journal of Pharmacology. 2001; 421: 109–114.