Original Articles

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Role of mast cell in hyperalgesic priming and the preventive effect of electroacupuncture on the transition from acute to chronic pain

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Abstract

Objective: Injury can lead to long-term changes that increase the sensitivity of afferent nerve endings to subsequent stimulation and pain can transition from acute to chronic. This phenomenon is known as hyperalgesic priming (HP). This study aimed to understand the mechanisms underlying the effect of electroacupuncture (EA) on HP and optimize acupoint selection for EA to prevent pain transition.

Methods: A rat HP model was established using sequential intraplantar injections of carrageenan (Cg) and prostaglandin E2 (PGE2). The pain thresholds were measured using von Frey filaments. EA on bilateral Zusanli (ST36) and Kunlun (BL60) was used to prevent pain transition. The number of mast cells in the ipsilateral hindpaw skin was determined using toluidine blue or fluorescence-labeled avidin staining. The protein expression levels of protein kinase C epsilon (PKCε) in the lumbar dorsal root ganglions (DRGs) were detected by western blotting 24 h after PGE2 injection. Serial pharmacological experiments were conducted to evaluate the relationship between mast cells and pain transition. Finally, EA on the bilateral ST36 and Chongyang (ST42) or a novel combination (ST36 and ST42 on the ipsilateral side, and ST36 and BL60 on the contralateral side) was used to prevent pain transition.

Results: Although EA applied to ST36 and BL60 alleviated acute pain induced by Cg injection, it failed to prevent the pain transition caused by PGE2 injection. Mast cell accumulation in the ipsilateral hind paw was observed 7 days after Cg injection. Furthermore, mast cell degranulation may be responsible for PKCε activation in the DRG, a marker of pain transition. EA significantly decreased the number of mast cells in the skin of the ipsilateral hind paw when applied at ST36 and ST42, but not when applied at ST36 and BL60. Furthermore, EA employed to ST36 and ST42 significantly reversed long-term hyperalgesia induced by PGE2 injection, even when administered before injection. However, EA did not alleviate acute pain caused by Cg injection. By using a novel acupoint combination, EA simultaneously alleviated acute pain and prevented pain transition.

Conclusions: Our study suggests that mast cells play a critical role in both HP and the transition from acute to chronic pain, whereas EA can prevent pain transition by decreasing the number of mast cells in the local tissue.

Keywords: Acupoints, Electroacupuncture, Hyperalgesic priming, Mast cell, PAR2

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Introduction

Acute pain is common after an injury. Fortunately, most instances of acute pain can be managed through interventions such as ice packs, nonsteroidal anti-inflammatory drugs, and other methods of pain relief. However, some individuals may continue to experience pain even after their injury has healed; this can serve as a precursor to chronic pain. Chronic pain has a complex pathogenesis, with an indeterminate molecular mechanism that impedes its clinical treatment. Blocking the transition from acute to chronic pain is considered an opportunity to develop novel therapeutics[1]. Over a decade ago, an animal model known as hyperalgesic priming (HP) was introduced as a tool to investigate the process by which acute pain transitions into chronic pain[2].

The scientific literature suggests pain transition involves two key phases: HP and pain transition[3]. Following pain transition, fundamental alterations may occur in neural circuits and/or phenotypes, diminishing the efficacy of therapies for acute pain[1,4]. Moreover, the pain transition

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is highly dependent on HP state. Exposure to inflammatory mediators sensitizes the peripheral terminals of the primary afferent nociceptors. This phenomenon is known as HP and determines whether other inflammatory mediators can produce long-lasting reductions in mechanical pain thresholds[5]. Therefore, two priorities should be geared toward developing more effective pain management methods[1]. First, acute pain and the mechanisms that contribute to chronic pain development should be blocked simultaneously, for instance, by regulating HP. Second, therapeutics should mimic or target endogenous pain-resolution mechanisms to reduce or potentially reverse chronic pain.

Based on the above suggestion, electroacupuncture (EA) has great potential as an effective therapeutic agent for pain relief. Scientific research indicated that EA activates endogenous opioid system, thereby mitigating various types of both acute and chronic pain[6,7], satisfying the second priority. Previous research conducted by our team indicates that EA inhibits protein kinase C epsilon (PKCε) activation within the ipsilateral dorsal root ganglion (DRG), effectively blocking pain transition[8,9]. Thus, EA can concurrently block acute pain and regulate pain transition. However, further research has indicated that although EA effectively regulates acute pain and pain transition, it may not prevent pain transition[8,9], suggesting that EA may not effectively regulate the mechanisms that promote the development of chronic pain. Because the therapeutic capabilities of EA depend on its stimulation parameters[7,10], further optimization of these parameters may facilitate the ability of EA to regulate hyperalgesia priming and prevent pain transition. Investigating this mechanism is imperative for optimizing EA parameters and bridging the gaps between advances in acupuncture therapeutics and our understanding of the hyperalgesia priming state.

Pain transition involves several peripheral neuronal receptors, including GABAAR[11], mGluR5[8], and protease-activated receptor 2 (PAR2)[12], which is a G protein-coupled receptor believed to contribute significantly to pain progression[13,14]. Serine proteases found in mast cells, such as tryptase, are natural ligands of PAR2[15]. Mast cells, which mainly exist in dermis of the skin and near nerves and blood vessels, help protect against physical, chemical, and pathological stress and are believed to contribute to chronic pain[16–18]. Inhibition of hind paw PAR2 blocked pain transition in a HP rat model[12], indicating that mast cells play a role in the transition from acute to chronic pain. Recent studies have proposed that pain caused by inflammation and tissue injury is not affected by the absence of mast cells[19,20]. These findings imply that the presence of mast cells in the skin may not influence the pain threshold but could affect the body's response to chemical stimuli, particularly prostaglandin E2 (PGE2). This state is likely to involve HP. Furthermore, PGE2, a typical pro-inflammatory cytokine, can cause mast cell degranulation by binding to its EP3 receptor[21]. This crucial mechanism may participate in the pain transition by releasing tryptase and activating PAR2. Mast cell degranulation is the initial step of acupuncture[22]. Given the involvement of mast cells in HP, EA may regulate the function of mast cells in local tissue, thereby preventing the transition of pain. This study aimed to examine the contribution of mast cells to the HP state and to determine whether EA can reduce mast cell numbers in the peripheral skin and prevent pain transition.

Methods

Animals

This study used adult male Sprague–Dawley rats (weighing 180–230 g) obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Animal Certificate No. SCXK(Hu)2013-0016). The rats were housed with *ad libitum* access to food and water in a controlled environment (25°C ± 2°C, 50% ± 10%) on a 12-hour light/dark cycle at the Zhejiang Chinese Medical University Laboratory Animal Center (SYXK(Zhe)2013-0184). This study was guided by the Regulations of the People's Republic of China on the Administration of Experimental Animals and approved by the Ethics Committee of Animal Center of Zhejiang Chinese Medical University (approval no. IACUC-20180319-12).

Drug preparation

Cg and PGE2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PKCε inhibitor PKCεV1-2 was purchased from Calbiochem, Millipore Sigma (Darmstadt, Germany). The PAR2 antagonist FSRLLY and mast cell agonist compound 48/80 (C48/80) were purchased from MCE (Merced, CA, USA). All drugs were dissolved in sterile saline and then diluted to the appropriate concentrations before injection (Cg: 1 mg/100 μL; PGE2: 100 ng/25 μL; PKCεV1-2: 1 μg/25 μL; FSRLLY: 10 μg/25 μL; C48/80 3 μg/25 μL). To deplete the mast cells in the skin, we administered C48/80, a 0.1% (w/v) solution in NS (normal saline), at a dose of 0.6 mg/kg, twice a day for three consecutive days and then doubled the dose on the fourth day as described in previous studies[23].

HP model

The method used to induce HP was consistent with that used in previous studies[9]. The rats were anesthetized, and the HP model was established by administering a 0.1 mL injection of Cg (first injection) followed by a 25 μL injection of PGE2 (second injection) in the same paw after 7 days. The sham HP group received the same volume (0.1 mL) of sterile saline (NS, 0.9% NaCl) instead of Cg. The model was successfully established when hyperalgesia induced by PGE2 injection lasted longer than 4 hours, according to a previous study[5]. Details of the drug used for the first and second injections in each group were described in the experimental design and group sections.

Mechanical withdrawal thresholds (MWTs)

Nociceptive behaviors were evaluated at specific time points throughout the study, including before the first injection (base) and 4, 24, 48, 72 hours, and 7 days after the first injection, as well as 1, 4, and 24 hours after the second injection. The experimenters have no

idea about the study conditions throughout the study. MWTs were assessed using the up-down method with von Frey filaments (Stoelting, IL, USA)[24]. The lateral plantar surface of the ipsilateral paw was stimulated with von Frey filaments ranging from 0.4 to 26 g (0.4, 0.6, 1, 2, 4, 6, 8, 15, and 26 g). The initial applied filament had a force of 4 g. Subsequently, a greater or lesser force filament was selected based on the negative or positive withdrawal response, respectively, and were as O or X. Outcomes were computed using a previously described function.

Administration of EA

EA intervention began 4 hours after the first injection and after the behavioral test. A customized retainer was used to gently immobilize rats, and then stainless-steel needles (0.18 mm × 13 mm) were inserted bilaterally into selective acupoints 5-mm deep. A series of acupuncture intensities (0.5, 1.0, and 1.5 mA) were increased every 10 minutes (total 30 minutes) using a HANS analgesic apparatus (LH-202H, Huawei Co., Ltd., Beijing, China) connected to the needles. EA was administered once per day at 2/100 Hz (2 and 100 Hz electric stimulus altered every 3 seconds) until the end of the experiment or otherwise in specific cases. The EA parameters were selected based on our previous studies[9,11]. For the same reason, the bilateral Zusanli (ST36) and Kunlun (BL60) were selected to regulate HP and prevent pain transition in Part I of this study. Based on the results of Part II, bilateral ST36 and Chongyang (ST42) were selected to prevent pain transition. Finally, ipsilateral ST36 and ST42 and contralateral ST36 and BL60 were selected to simultaneously relieve acute pain and regulate HP.

Sham EA was performed by inserting needles subcutaneously (1-mm-depth) into the ST36 and BL60 acupoints, under the same stimulator setting, without delivering electrical stimulation.

Staining of mast cells

Rats were euthanized after completing the MWTs assessment according to the experimental design. Rats were anesthetized with 2% pentobarbital sodium (40 mg/kg, intraperitoneally). Then, the rats were quickly perfused with 0.9% NaCl (4°C), followed by perfusion with 4% paraformaldehyde in 0.1 M PBS for prefixation. The dorsal skin of the ipsilateral paw in the PGE2 injection area was removed, fixed in 4% paraformaldehyde for 3 hours at 4°C, then transferred to 15% and 30% sucrose for dehydration and preserved in a −80°C freezer. Skins were transversely sectioned (10 μm) with a cryostat and dried at room temperature (RT) for 30 minutes. Mast cells were identified using 1% toluidine blue staining or fluorescence-labeled avidin[16]. Mast cells were counted in low-power fields (magnification, ×100). Degranulated mast cells were counted in high-power fields (magnification, ×400). Degranulated mast cells were defined as those with reduced granule density throughout the entire cell[25]. The ratio of degranulated mast cells was calculated by dividing the number of degranulated mast cells by the total number of mast cells on each slice.

Western blotting

Western blotting procedure was conducted as outlined in our previous study[11]. Total protein was extracted from L4-L6 DRG tissues using RIPA lysis buffer (Beyotime, China) supplemented with 1% phenylmethylsulfonyl fluoride (Beyotime) and a comprehensive protease/phosphatase inhibitor cocktail (Applygen, China). The protein concentration was quantified using the bicinchoninic acid method. Subsequently, 20 μg protein samples were loaded onto 5% SDS-PAGE gels and electrophoretically transferred onto PVDF membranes (Bio-Rad, USA). Prior to antibody incubation, the membranes were blocked with 5% low-fat milk for 1 hour at RT. They were then incubated overnight at 4°C with primary antibodies specific for anti-PKCε (1:1,000 in 5% normal goat serum, NGS; Abcam, USA) or anti-β-actin (1:1,000 in 5% NGS; Abcam). Following this, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:5,000; Abcam) for 1 hour at RT. Following this, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; Abcam) for 1 hour at RT. For visualization, an enhanced chemiluminescence kit (Pierce, USA) was utilized, and the luminescent signals were captured using an ImageQuant LAS 4000 imaging system (GE, USA). The band densities were quantitatively analyzed using ImageQuant TL 7.0 analysis software (GE). The mean expression level of the target proteins in animals from the first group was set to 1, and the relative expression levels of the target proteins in all other animals were normalized to this baseline value.

Experimental design and group

This study aimed to optimize the stimulation parameters of EA to prevent pain transition effectively. All experimental designs are shown in Figure 1.

Part I

This study aimed to investigate the effect of EA on pain transition in HP model rats at different time points. Initially, we examined the effects of EA on the MWTs and the expression level of PKCε to demonstrate its role in regulating the transition from acute to chronic pain. Four randomized groups were created for this part of the study, namely NS + PGE2 (injection of PGE2 followed NS), Cg + PGE2 (injection of PGE2 followed Cg), Cg + PGE2 + EA (rats with Cg + PGE2 injection and treated with EA stimulation), and Cg + PGE2 + sEA (rats with Cg + PGE2 and given sham EA stimulation) (Figure 1A). Subsequently, we attempted to prevent pain transition by applying EA stimulation prior to the onset of chronic pain. For this part of the study, we randomly divided the rats into two groups: Cg + PGE2 and Cg + PGE2 + EA I (rats that were subjected to EA stimulation only between their first and second injection of Cg + PGE2) (Figure 1B). The effect of EA I on MWTs was tested.

Part II

This study aimed to determine the peripheral mechanism underlying HP. Initially, we examined the MWT of the

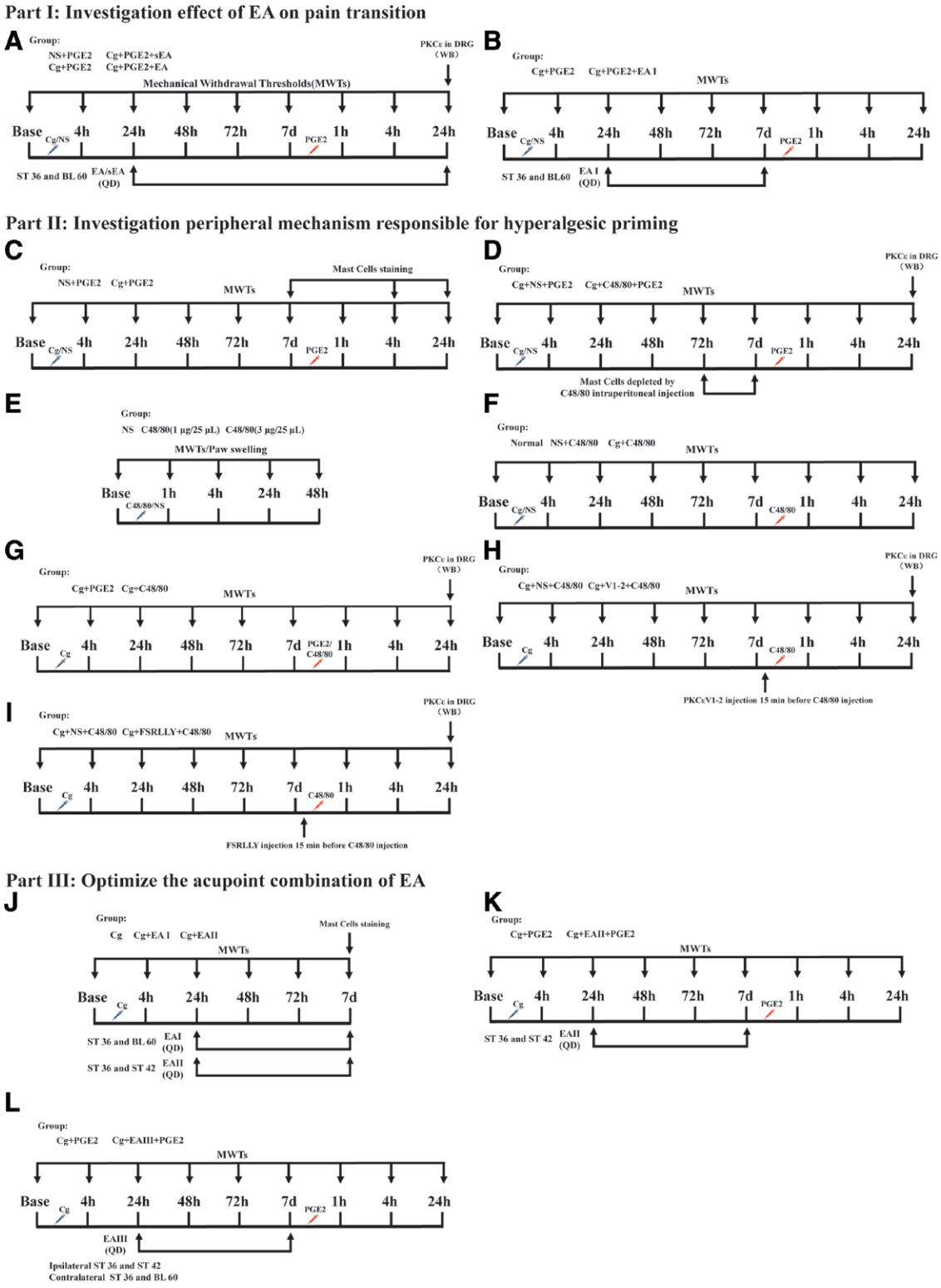

Figure 1. Graphic representation of the overall experimental design. Part I: This aimed to investigate the effect of EA on pain transition in hyperalgesia priming model rats when EA was applied during different time points. The study was divided into two parts. Part II: This aimed to determine the peripheral mechanism responsible for HP. The study was divided into seven parts. Part III: The purpose of this study was to optimize the acupoint combination of EA according to the results from Part II. The study was divided into three parts. BL60: Kunlun; C48/80: Compound 48/80; Cg: Carrageenan; DRG: Dorsal root ganglion; EA: Electroacupuncture; NS: Normal saline (0.9% NaCl); PGE2: Prostaglandin E2; PKCε: Protein kinases C epsilon; ST36: Zusanli; ST42: Chongyang.

rats to confirm the establishment of HP model. In this part of our study, we randomly divided the rats into two groups, NS + PGE2 and Cg + PGE2 (Figure 1C). We investigated the number and degranulation of mast cells in the dorsal skin of the ipsilateral hind paws to identify the possible role of mast cells in HP. We selectively depleted mast cells to confirm their role in PGE2-induced long-term hyperalgesia. We divided the rats into two groups: Cg + NS + PGE2 and Cg + C48/80 + PGE2 (Figure 1D). Additionally, we examined the effect of mast cell depletion

on PKCε expression to establish the relationship between PKCε and mast cells. To confirm the role of mast cells in HP, we injected C48/80 followed by Cg to establish a HP model. We tested the duration of inflammation and inflammatory induced by C48/80 intraplantar injection. We randomly divided the rats into three groups: NS, Cg (1 μg/25 μL) and NS, Cg (3 μg/25 μL) (Figure 1E). We randomly divided the rats into three groups: normal, NS + C48/80, and Cg + C48/80 groups (Figure 1F). Finally, we demonstrated the importance of the PAR2-PKCε pathway in the pain transition induced by C48/80 by using the selective antagonists of PKCε and PAR2. In this part, we randomly divided the rats into the following groups: Cg + PGE2 and Cg + C48/80 groups (Figure 1G); Cg + NS + C48/80 and Cg + V1-2 + C48/80 groups (injection of PKCεV1-2 before C48/80) (Figure 1H); Cg + NS + C48/80 and Cg + FSRLLY + C48/80 groups (injection of FSRLLY before C48/80) (Figure 1I). We also explored differences in MWTs and PKCε expression between the groups in this part.

Part III

This study aimed to optimize the acupoint combination of EA according to the mechanism of mast cells to prevent pain transition. During this part of the study, EA was only administered between the first and second injection. Initially, we optimized the acupoint combination to reduce the number of mast cells in the dorsal skin of the ipsilateral hind paw. For this part of the study, we randomly divided the rats into three groups: Cg, Cg + EA I (administered EA at ST36 and BL60), and Cg + EA II (administered EA at ST36 and ST42) (Figure 1J). We investigated the number of mast cells in the dorsal skin of the ipsilateral hind paws. We investigated the effects of EA II on MWTs after PGE2 injection to demonstrate that EA is capable of preventing pain transitions. For this part of the study, we randomly divided the rats into two groups: Cg + PGE2 and Cg + EA II + PGE2 (Figure 1K). Finally, we attempted to alleviate acute pain while simultaneously preventing pain transition. For this part of the study, we randomly divided the rats into three groups: Cg + NS, Cg + PGE2, and Cg + EA III + PGE2 (EA was applied on ST36 and ST42 on the ipsilateral side and ST36 and BL60 on the contralateral side) (Figure 1L). The effect of EA III on MWTs was tested.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). For comparing two independent samples, the Student *t* test was applied. When comparing three or more samples, an analysis of variance was conducted, followed by Bonferroni multiple comparison test. Statistical significance was determined at a *P* value of less than 0.05.

Results

EA regulated pain transition but failed to prevent it in the HP rats

Figure 2A illustrates the time points of MWTs and EA interventions. Consistent with previous studies[26], EA administered at ST36 and BL60 significantly alleviated acute pain caused by Cg injection and chronic pain induced by Cg + PGE2 injection (Figure 2B). Furthermore, EA reduced the expression level of PKCε in the ipsilateral lumbar DRG 24 hours after PGE2 injection, confirming the successful administration of EA (Figure 2C). These findings align with the results reported in our previous study; therefore, we believe that EA successfully regulated pain transition[9]. To assess the impact of EA on the HP state, EA was administered before the PGE2 injection (EA I in Figure 1A) during the acute pain and HP phase. While EA alleviated acute pain caused by Cg injection, it did not increase MWTs in rats receiving further PGE2 injection at 4 and 24 hours post-injection (Figure 2D). Thus, the analgesic effect of EA observed in this study was distinct from its preventive effect on HP rats. We speculate that the EA applied during the HP phase failed to prevent pain transition.

Mast cells contribute to the HP state and pain transition

To investigate the potential effect of EA in preventing pain transition, we first examined the mechanisms underlying HP. As previous studies have suggested, activation of PAR2 is sufficient to induce a transition from acute to chronic pain[12]. Mast cells are the primary source of tryptase, a PAR2 ligand, in the skin. Therefore, we explored whether mast cells are involved in pain transition and hyperalgesia priming (Figure 3A). A hyperalgesia priming rat model was established and the MWTs of the model rats were significantly lower than those of the control group 24 hours after PGE2 injection (Figure 3B). Mast cells were observed in the dorsal skin of the ipsilateral hind paw (Figure 3C), and the number of mast cells and the level of mast cell degranulation did not change 24 hours after PGE2 injection (Figure 3C, D). We evaluated the role of mast cells in the HP state by measuring the number of mast cells in the skin 7 days after Cg injection. We found that the number of mast cells significantly increased 7 days after Cg injection (Figure 3E, F), although the MWTs recovered at that time point [Supplementary Figure 1A, http://links.lww.com/AHM/A143]. However, there was no significant difference in the level of mast cell degranulation between the two groups (Figure 3E, F). We also observed mast cells in the skin 4 hours after PGE2 injection (Figure 3G) [Supplementary Figure 1B, http://links.lww.com/AHM/A143] and found that the number of mast cells significantly decreased after PGE2 injection, with considerable mast cell degranulation (Figure 3G, H). These results suggest that the number of mast cells increases after an acute inflammatory response and may contribute to pain transition within a short period.

To further validate the function of mast cells in pain transition and hyperalgesia priming, we depleted mast cells from the skin using C48/80. Four days after the intraperitoneal injection of C48/80, the number of mast cells decreased significantly in the dorsal skin of the ipsilateral hind paw (Figure 4A, B). Intraperitoneal injection of C48/80 did not affect the pain threshold of rats, and there was little difference between the two groups 7 days after Cg injection (Figure 4C). Importantly, rats that received C48/80 intraperitoneal injection did not display long-term

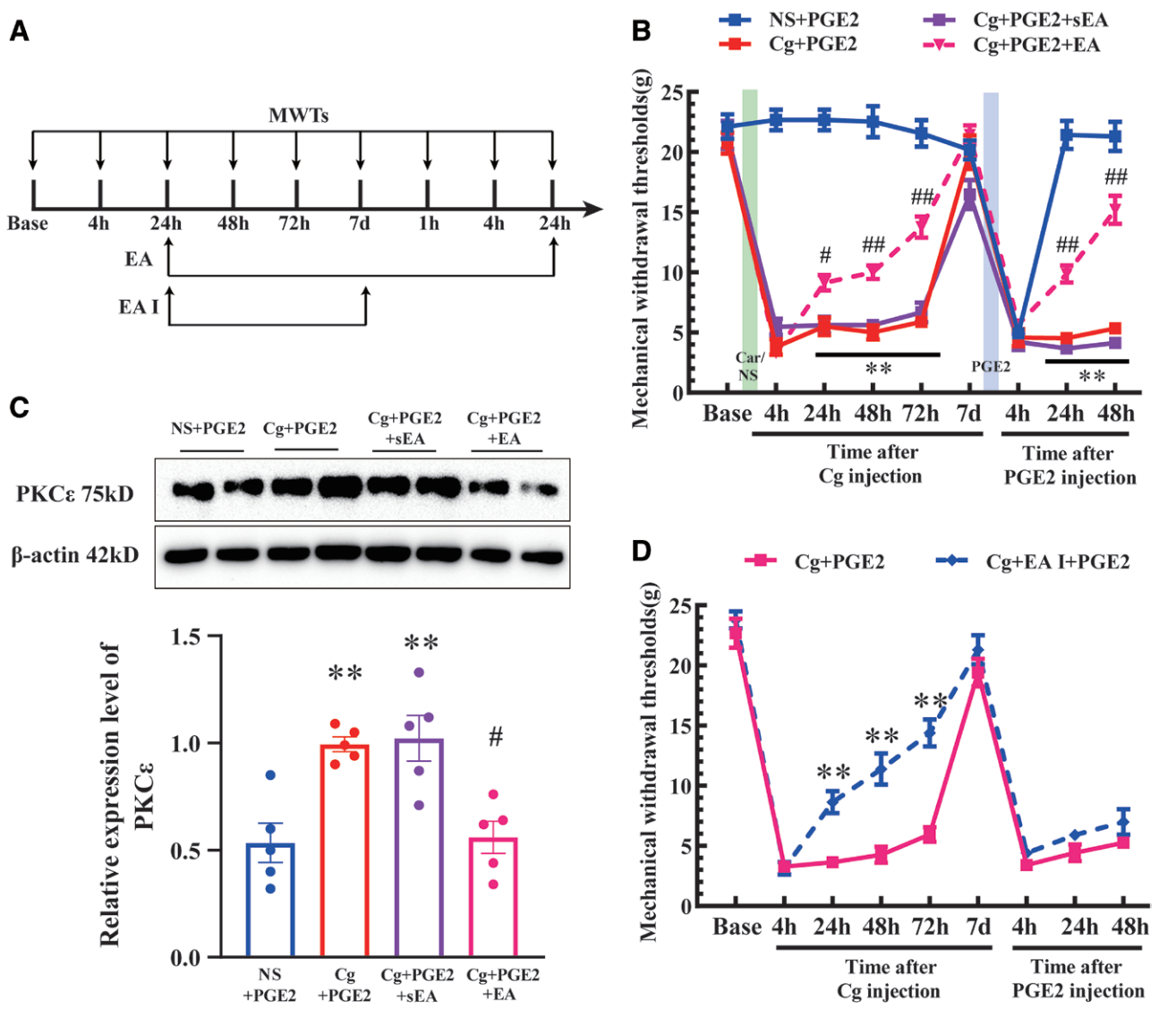

Figure 2. EA applied at ST36 and BL60 alleviate the acute pain and regulate transition from acute to chronic pain. (A) The experimental protocol, with timing of EA and EA I treatments and MWTs measurements. (B) Cg + PGE2 injection established a hyperalgesic priming model, and EA applied at ST36 and BL60 significantly increased the MWTs of rats before and after PGE2 injection. n = 6; (C) EA decreased the expression level of PKCε in lumbar DRG. The quantification of the western blot results and a representative Western blot showing PKCε protein isolated from the L4–L6 DRG 24 h after PGE2 injection. n = 5. (D) EA applied at ST36 and BL60 before PGE2 injection failed to regulate the MWTs of model rats after PGE2 injection. n = 6. Compared with NS + PGE2 group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with Car + PGE2 group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Data are shown as mean ± SEM. BL60: Kunlun; Cg: Carrageenan; DRG: Dorsal root ganglion; EA: Electroacupuncture; MWT: Mechanical withdrawal thresholds; NS: Normal saline (0.9% NaCl); PGE2: Prostaglandin E2; PKCε: Protein kinases C epsilon; SEM: Standard error of the mean; ST36: Zusanli.

hyperalgesia following PGE2 intraplantar injection, even after Cg injection (Figure 4C). However, PGE2 was still able to produce acute pain (Figure 4C). As expected, the PKCε expression level in the lumbar DRG was decreased with MWTs increasing 24 hours after PGE2 injection (Figure 4D). We also investigated whether mast cell stimulation induces long-term hyperalgesia after Cg injection. Prior to using C48/80 injection following Cg to recreate the HP model, we observed dose-dependent hyperalgesia and inflammation induced by a single intraplantar injection of C48/80 (Figure 4E–H). A single intraplantar injection of C48/80 was only able to produce a short duration of inflammation (<4 hours), regardless of dose (Figure 4E). The strongest inflammation was induced 1 hour after C48/80 injection (Figure 4E, F). Both C48/80 concentrations induced acute hyperalgesia only 1 hour after injection, which was consistent with the paw swelling results (Figure 4G). No significant differences in acute pain were observed when the different concentrations of C48/80 used for induction were compared (Figure 4H). According to the results, a concentration of 1 μg/25 μL C48/80 was used to produce the hyperalgesia priming model. As expected, C48/80 intraplantar injection following NS injection produced only acute pain lasting less than 4 hours, whereas C48/80 injection following Cg injection produced long-term hyperalgesia lasting longer than 24 hours (Figure 4I). These results indicate that mast cell accumulation and degranulation in the local skin may be the mechanisms underlying HP model type I.

PAR2-PKCε pathway is involved in the pain transition induced by mast cell degranulation

Previous studies showed that activation of PKCε in DRG is the key mechanism underlying pain transition[27]. To further ascertain the part played by mast cells in the

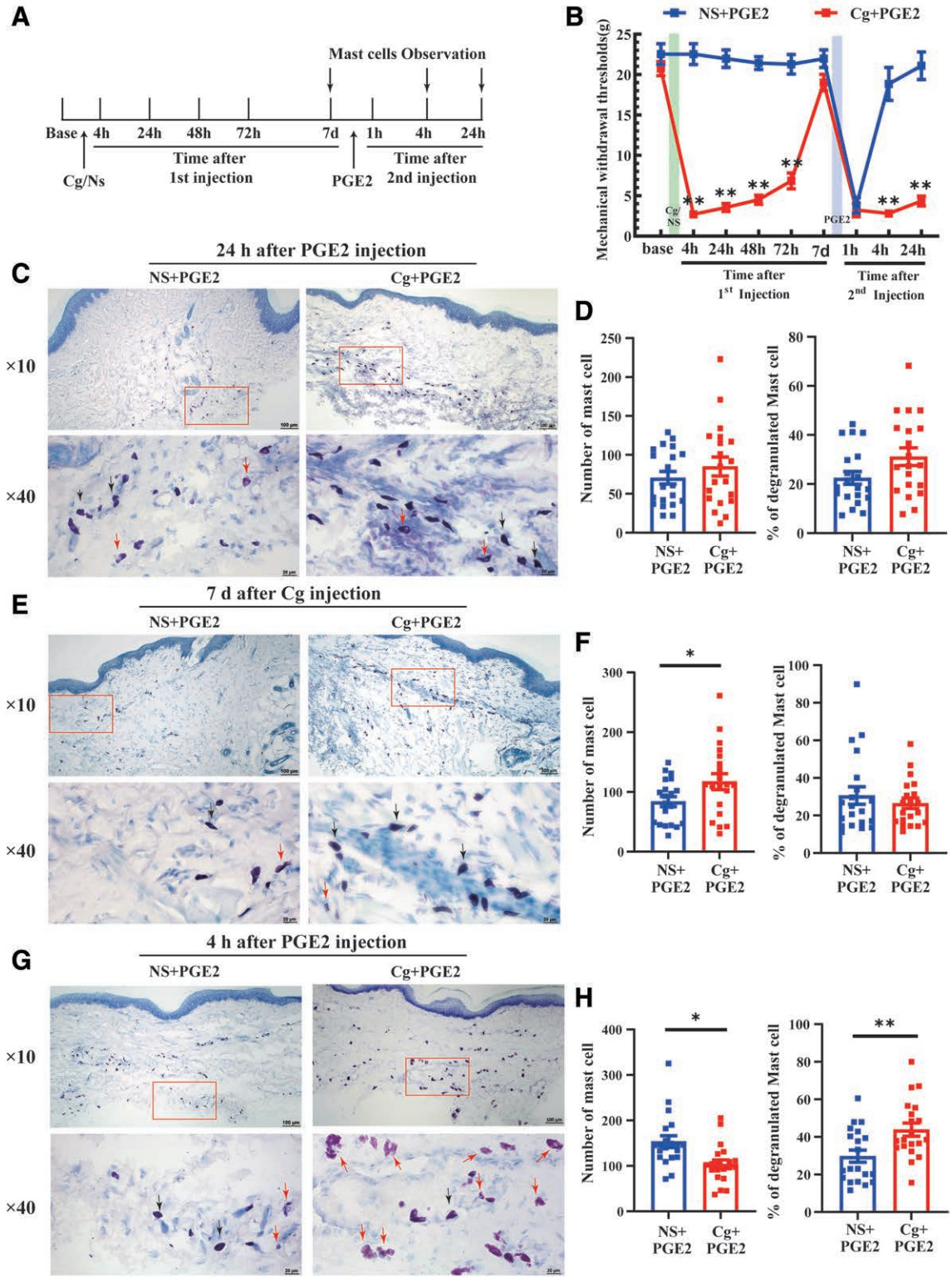

Figure 3. Mast cells accumulated in the dorsum skin of the ipsilateral hind paw after Cg injection have been activated by PGE2. (A) The experimental protocol, with timing of Cg and PGE2 injection and mast cells observation. (B) Cg + PGE2 injection established a hyperalgesic priming model, and the MWTs of rats decreased after Cg and PGE2 injection. $n = 6$; (C) Mast cell staining by toluidine blue in the dorsum skin of ipsilateral hind paw 24 h after PGE2 injection. (D) Cg and PGE2 injection did not change the number and degranulation level of mast cells in the skin. (E) Mast cell staining by toluidine blue in the dorsum skin of ipsilateral hind paw 7 days after Cg injection. (F) Cg injection increased the number of mast cells in the skin but not the degranulation level. (G) Mast cell staining by toluidine blue in the dorsum skin of ipsilateral hind paw 4 h after PGE2 injection. (H) PGE2 injection decreased the number of mast cells in the skin and increased the degranulation level. *$P < 0.05$, **$P < 0.01$. $n = 20$ slices from four rats. Scale bar is 100 and 20 μm under ×10 and ×40 eyepiece. Data are illustrated as mean ± SEM. Cg: Carrageenan; MWT: Mechanical withdrawal thresholds; NS: Normal saline (0.9% NaCl); PGE2: Prostaglandin E2; SEM: Standard error of the mean.

pain transition, the PKCε protein expression level was investigated in L4-L6 DRG. Similar to the Cg + PGE2 injection, sequential intraplantar injection of Cg and C48/80 produced long-term hyperalgesia (Figure 5A) accompanied by an elevated expression level of PKCε in ipsilateral L4-L6 DRG (Figure 5B). Administration

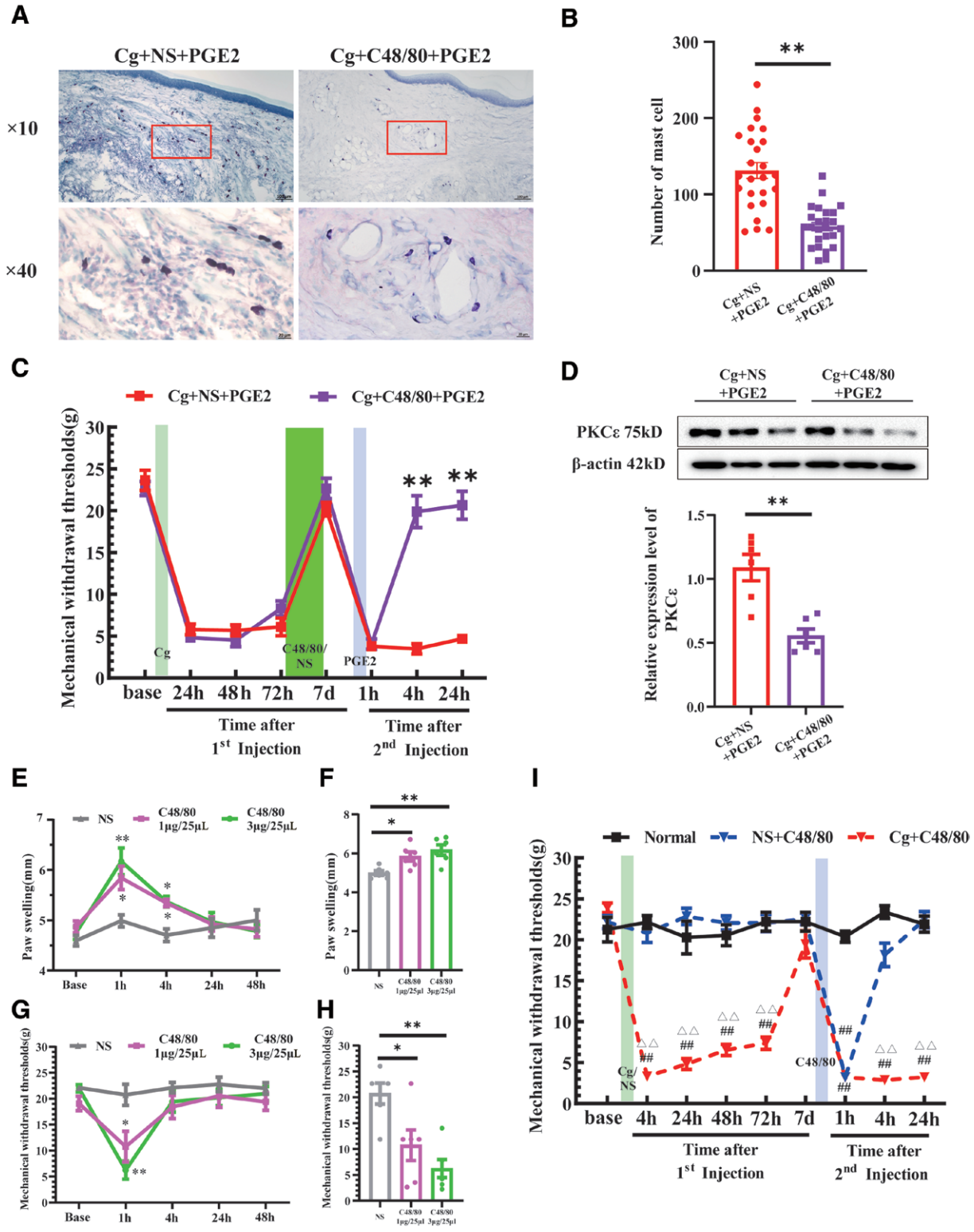

Figure 4. Mast cells accumulation and degranulation in the local skin are involved in the hyperalgesia priming and pain transition. (A) Mast cell staining in the dorsum skin of ipsilateral hind paw 4 days after C48/80 depletion. (B) Intraperitoneal injection of C48/80 decreased the number of mast cells in the dorsum skin of ipsilateral hind paw. $n = 20$ slices from 4 rats. (C) Mast cells depletion by C48/80 prevent the long-term hyperalgesia induced by PGE2 injection but not acute pain. $n = 6$. (D) Mast cell depletion by C48/80 decreased the expression level of PKCε in lumbar DRG 24 h after PGE2 injection. The quantification of the western blot results and a representative western blot showing PKCε protein isolated from the L4–L6 DRG 24 h after PGE2 injection. $n = 6$. (E) Intraplantar injection of C48/80 induced dose-independent acute inflammation in the hind paw. $n = 6$. (F) The inflammation induced by C48/80 reached the peak 1 h after injection. $n = 6$. (G) Intraplantar injection of C48/80 induced dose-independent acute pain in the hind paw. $n = 6$. (H) The acute pain induced by C48/80 reached the peak 1 h after injection. $n = 6$. (I) Cg + C48/80 injection is able to recreate the hyperalgesic priming model in rats. $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$; compared with the normal group, $^{\#\#}P < 0.01$; compared with NS + C48/80 group, $^{\triangle\triangle}P < 0.01$. Data are shown as mean ± SEM. C48/80: Compound 48/80; Cg: Carrageenan; DRG: Dorsal root ganglion; NS: Normal saline (0.9% NaCl); PGE2: Prostaglandin E2; PKCε: Protein kinases C epsilon; SEM: Standard error of the mean.

of a selective PKCε antagonist, PKCεV1-2, significantly reversed the long-duration hyperalgesia induced by Cg injection following C48/80 (Figure 5C). The antagonist significantly inhibited both the hyperalgesia and PKCε activation in the lumbar DRG concurrently (Figure 5D). These findings are consistent with previous results in a HP model established by Cg + PGE2 injection[28]. The results indicated that mast cells' degranulation-induced long-duration hyperalgesia is dependent on PKCε activation in the lumbar DRG.

However, C48/80 can initiate calcium influx and directly activate neurons[29]. Therefore, C48/80 may not activate PKCε and induce pain transition through mast cell stimulation. FSRLLY (a PAR2 antagonist) was used to investigate whether C48/80 induced pain transition was independent of mast cell degranulation. FSRLLY injection prevented long-duration hyperalgesia induced by the Cg + C48/80 injection (Figure 5E); however, it failed to alleviate the acute pain induced by C48/80 (Figure 5E). The expression level of PKCε was also explored. Blocking peripheral PAR2 inhibited PKCε activation in the ipsilateral lumbar DRG (Figure 5F). These results indicate that PAR2 plays a pivotal role in the long-duration hyperalgesia induced by C48/80 injection. Moreover, PAR2-PKCε pathway was implicated in the pain transition elicited by C48/80 administered.

EA decreased the number of mast cells in the local skin and prevented the pain transition

As mentioned earlier, mast cell accumulation may be the mechanism underlying HP. EA should regulate the number of mast cells in the local skin to alter the HP state and prevent the transition of pain. Although the EA applied at bilateral ST36 and BL60 alleviate acute and chronic pain and inhibit PKCε activation, it failed to decrease the number of mast cells in the ipsilateral hind paw skin (Figure 6A–D). Previous studies have hypothesized that acupuncture stimulates mast cells through limited spatial effect, such as inducing muscle contraction[30]. Another special acupoint combination, ST36 and ST42, was selected to simultaneously produce analgesic effects and decrease the number of mast cells. As shown in Figure 6B, EA administered at bilateral ST36 and ST42 did not alleviate the acute pain induced by Cg injection. However, EA administered at bilateral ST36 and ST42 significantly decreased the number of mast cells in the dorsal skin of the ipsilateral hind paw 7 days after Cg injection (Figure 6D). More importantly, the PGE2 injection produced long-term hyperalgesia, which was reversed when EA was administered only at ST36 and ST42 before the PGE2 injection (Figure 6E). All results indicated that EA prevented pain transition when the correct selection of stimulation acupoints was used. However, effective pain management methods should be developed to alleviate acute pain. As the central nervous system also plays a pivotal role in EA analgesia, a special acupoint combination was selected where ST36 and ST42 were applied on the ipsilateral side to decrease the number of mast cells, and ST36 and BL60 were applied on the contralateral side to alleviate acute pain. The MWTs results are presented in Figure 6F. EA not only alleviated the acute pain induced by Cg injection but also prevented the long-duration hyperalgesia produced by Cg + PGE2 injection.

Discussion

Here, we present results that identify mast cells as crucial factors in inflammation-induced hyperalgesia priming and the transition from acute to chronic pain. Using a type I HP model, we found that EA stimulation could both prevent and regulate pain transition, depending on acupoint selection.

Previous research on EA has not identified significant interventions to prevent pain transition[9,11]. The acupuncture theory postulates that EA efficacy is affected by three factors: the pathological state of the body, stimulatory parameters, and acupoint selection. In the HP model, the stimulation parameters of EA, such as frequency, current intensity, and acupoint selection, can be further optimized. Inflammation involves complex biological responses of the somatosensory, immune, autonomic, and vascular systems. To tailor the acupoint selection for EA to prevent pain transition, primary afferent nerve responses to inflammation must first be understood in terms of how they establish a HP state.

Inflammation is the body's natural response to injury and infection and is always accompanied by pain, which serves as a crucial protective mechanism. Previous studies have indicated that exposure of the peripheral terminals of primary afferent nociceptors to inflammatory mediators (such as PGE2) can cause a long-lasting reduction in mechanical pain thresholds, which is determined by pre-exposure to other inflammatory mediators (eg, Cg)[5]. Given that functional changes in sensory neurons, which are responsible for allodynia and hyperalgesia, and the pain threshold of mammals exposed to inflammation, do not decrease before PGE2 injection, we hypothesized that non-neuronal cells may play a role in the hyperalgesia priming state. Mast cells are key immune cells and effectors in the inflammatory process and serve as a critical link between the nervous and immune systems[31]. The main mode of communication between mast cells and neurons is believed to involve mast cell degranulation and cytokine release. The release of cytokines such as tumor necrosis factor α, interleukins, and the CCL family from mast cells has been extensively documented as contributing to both inflammatory and neuropathic pain[32]. Previous studies have documented that PGE2 positively regulates mast cell degranulation *via* its EP3 receptor[21]. These results indicated that mast cells may be involved in PGE2-induced long-term hyperalgesia. We observed that mast cells are recruited to the local skin through inflammatory stimulation, and their numbers remain elevated even in the absence of ongoing inflammation. Importantly, we found that the accumulation of mast cells without stimulation or degranulation does not affect the pain threshold of rats, which is consistent with a previous study[19]. This phenomenon was also observed 24 hours after PGE2 injection, where model rats had significantly lower MWTs than sham model rats, despite having a comparable number of mast cells in the local skin. We hypothesized that the accumulation of mast cells may be the mechanism underlying the HP state and that mast cell degranulation likely serves as the primary

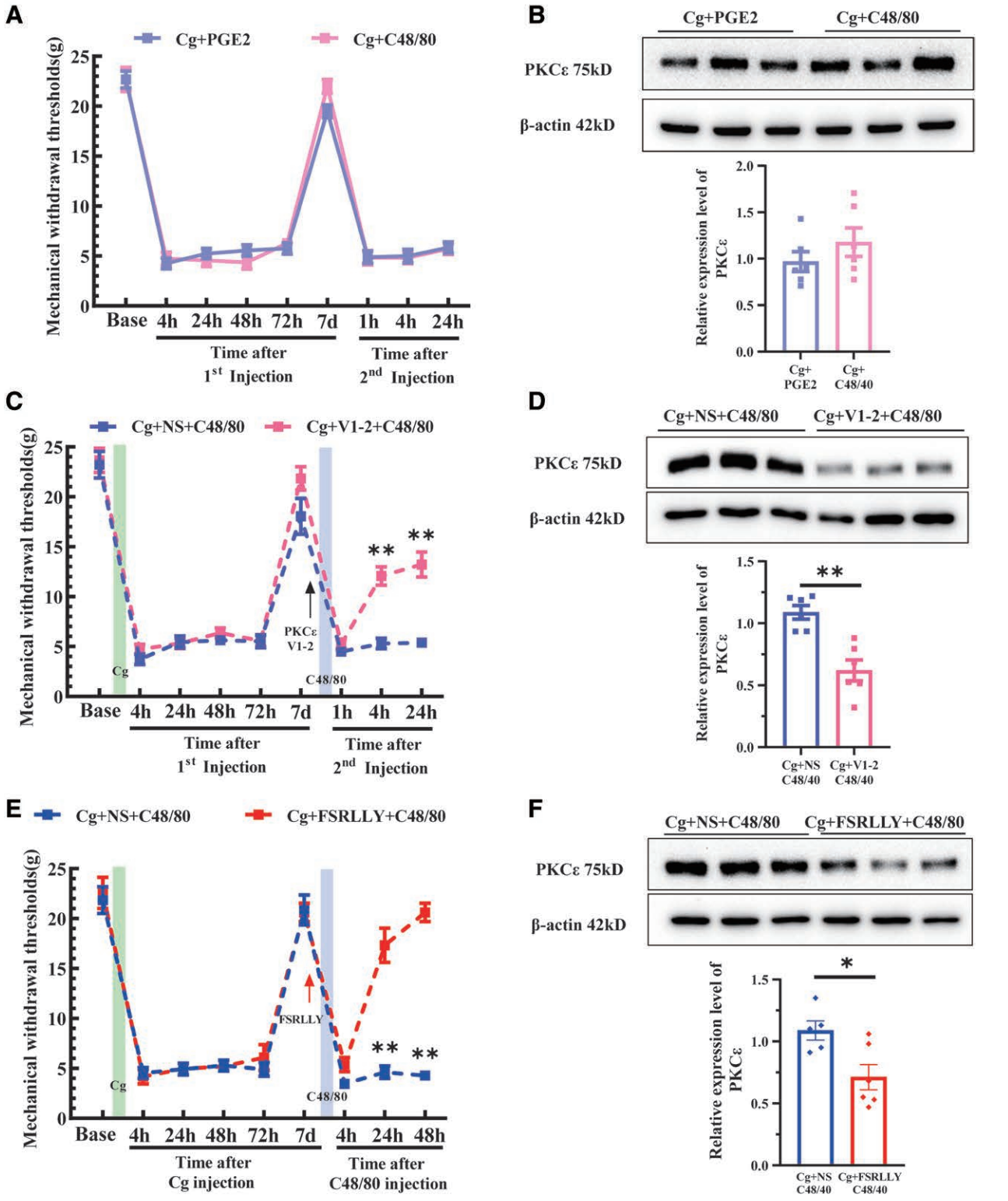

Figure 5. PAR2-PKCε pathway is involved in the pain transition induced by mast cell degranulation. (A) Intraperitoneal injection of C48/80 following Cg is able to recreate the hyperalgesic priming model. $n = 6$. (B) Intraperitoneal injection of C48/80 following Cg increased the expression level of PKCε in the lumbar DRG as Cg + PGE2 injection. The quantification of the western blot results and a representative western blot showing PKCε protein isolated from the lumbar DRG 24 h after C48/80 injection. $n = 6$. (C) Inhibition PKCε activation reversed the long-term hyperalgesia induced by Cg + C48/80 injection. $n = 6$. (D) Antagonist PKCεV1-2 reduced the expression level of PKCε in lumbar DRG. The quantification of the western blot results and a representative western blot showing PKCε protein isolated from the lumbar DRG 24 h after C48/80 injection. (E) Blocking PAR2 prevents the long-term hyperalgesia induced by Cg + C48/80 injection. $n = 6$. (F) Blocking PAR2 decreased the expression level of PKCε in the lumbar DRG. The quantification of the western blot results and a representative western blot showing PKCε protein isolated from the lumbar DRG 24 h after C48/80 injection. $n = 6$. $^*P < 0.05$, $^{**}P < 0.01$. Data are shown as mean ± SEM. C48/80: Compound 48/80; Cg: Carrageenan; DRG: Dorsal root ganglion; FSRLLY: A PAR2 antagonist; NS: Normal saline (0.9% NaCl); PGE2: Prostaglandin E2; PKCε: Protein kinases C epsilon; SEM: Standard error of the mean; V1-2: PKCεv1-2, a PKCε antagonist.

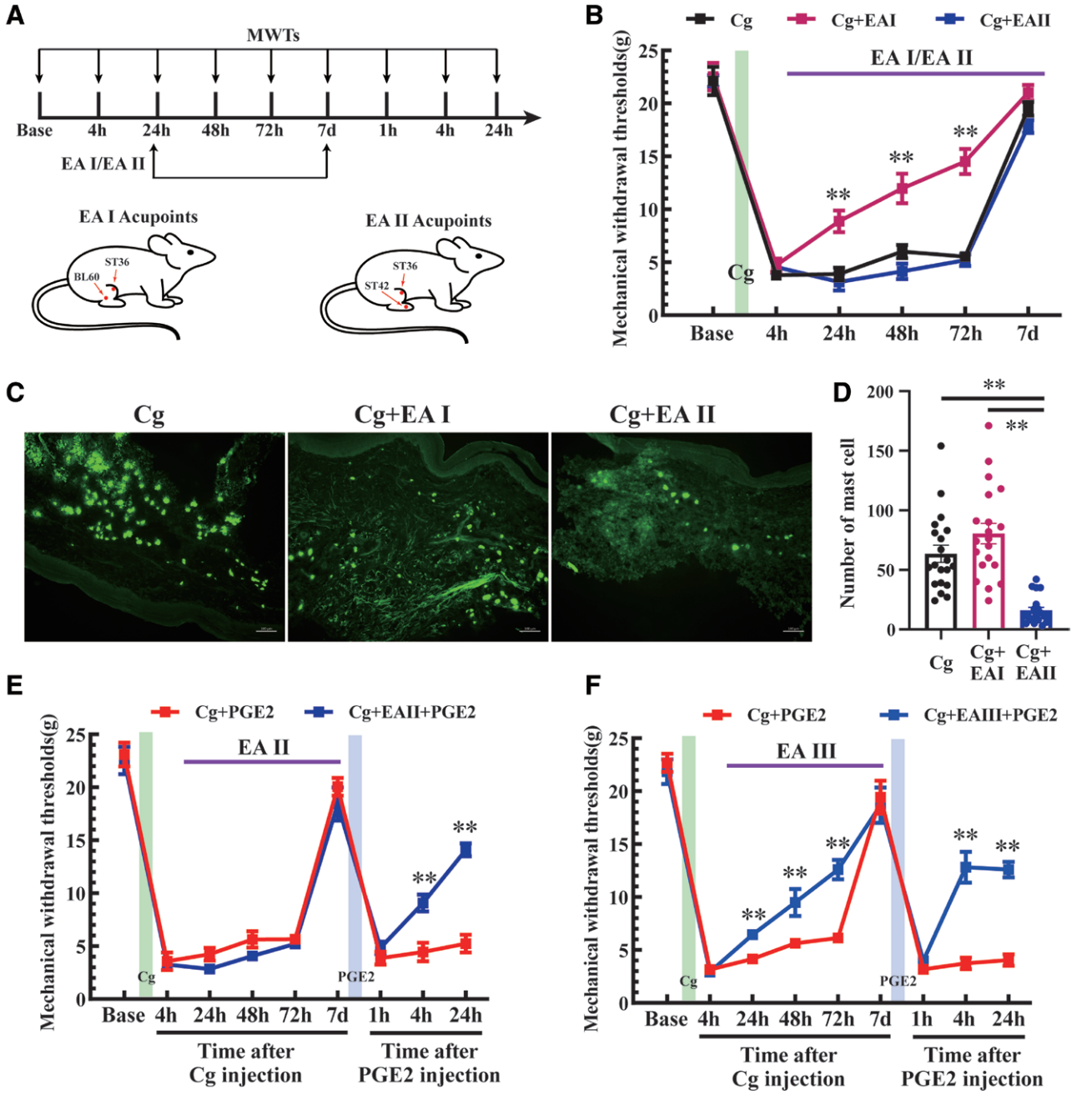

Figure 6. EA decreased the number of mast cells in the local skin and prevented pain transition. (A) The experimental protocol, with the timing of EA I and EA II treatments and MWT measurements, and the acupoint combination used in EA I and EA II treatments. (B) EA I (applied at ST36 and BL60) but not EA II (applied at ST36 and ST42) significantly increased the MWTs of rats after Cg injection. $n = 6$; (C) Mast cell staining by fluorescence-labeled avidin in the dorsum skin of ipsilateral hind paw 7 days after Cg injection. (D) EA II but not EA I decreased the number of mast cells in the skin. (B) EA I (applied at ST36 and BL60) but not EA II (applied at ST36 and ST42) significantly increased the MWTs of rats after Cg injection. $n = 20$ slices from four rats; (E) EA II significantly increased the MTSs of rats after PGE2 injection but not before, even if it was administered before PGE2 injection. $n = 6$; (F) EA III simultaneously alleviate the acute pain and regulate hyperalgesic priming induced by Cg injection. $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$. Data are shown as mean ± SEM. Cg: Carrageenan; EA: Electroacupuncture; MWT: Mechanical withdrawal thresholds; PGE2: Prostaglandin E2; SEM: Standard error of the mean.

trigger of long-term hyperalgesia. As expected, pharmacological depletion of mast cells prevented pain transition in HP model rats. Moreover, we observed that after 12 days of Cg exposure, PGE2 injection failed to induce long-term hyperalgesia (data not shown). We believe this may be due to the fact that mast cells are no longer recruited to the local skin after inflammation has subsided, and the number of mast cells has returned to normal levels. Furthermore, this phenomenon may partially explain why chronic pain is not induced by repeated injuries or inflammatory stimuli in humans.

Another reason for our hypothesis is that PAR2 has long been recognized as a critical receptor that contributes to pain processing[33,34], including pain transition[12]. Protease release from mast cells is believed to cause inflammatory pain *via* activation of PAR2[35]. Our findings demonstrate that long-term hyperalgesia induced by C48/80 is dependent on both PKCε and PAR2 activation. Moreover, PAR2 plays a pivotal role in the PKCε activation induced by C48/80. Previous studies have reported that PAR2 activation is related to PKCε activation induced by PGE2[36], as well as TRPV1 sensitization[37].

Furthermore, inhibition of PAR2 was sufficient to block pain transition caused by Cg + PGE2 injections. Our previous results showed that PKCε-TRPV1 pathway is involved in the pain transition[9]. Furthermore, the PAR2-PKCε-TRPV1 pathway is involved in inflammatory pain[38]. All the results indicate that mast cells induce pain transition *via* the PAR2-PKCε-TRPV1 pathway in the lumbar DRG.

Tryptase, a PAR2 ligand, is predominantly released from degranulating mast cells in the local skin. Thus, we indicate that mast cell activation and tryptase release contribute to the activation PKCε in the lumbar DRG and pain transition. The HP model was successfully replicated using Cg and mast cell degranulator C48/80. Some researchers have reported that C48/80 may induce hyperalgesia through the direct activation of peripheral neurons by initiating Ca^{2+} influx[29]. In this study, we have shown that Cg + C48/80 induced long-term hyperalgesia by activating PKCε, which is in alignment with the Cg + PGE2 protocol. Furthermore, PKCε activation was independent of calcium ion concentration level[39]. Thus, we hypothesize that C48/80 does not induce pain transition or activate PKCε through Ca^{2+} influx.

Our results support the hypothesis that mast cell accumulation is the underlying mechanism of HP. Our results showed that EA stimulation at the acupoints BL60 and ST36 did not decrease the number of mast cells in the dorsal skin of the ipsilateral hind paw. Furthermore, the current intensity and frequency mainly determine the localization of the EA effect in the nervous system[10]. Therefore, we hypothesized that changing only the current intensity and frequency of EA might not be sufficient to improve its preventive effects on pain transition. We selected acupoint ST42 to regulate the accumulation of mast cells, located close to the site of PGE2 injection. Administering EA at ST36 and ST42 significantly reduced the number of mast cells on the dorsal skin of the hind paw and improved the MWTs of rats after PGE2 injection, even when EA was applied before PGE2 injection. However, EA stimulation at ST36 and ST42 failed to alleviate the acute inflammatory pain induced by the Cg injection, possibly because EA stimulation at ST42 was excessively painful and produced an analgesic effect. We selected ST36 and ST42 on the ipsilateral side and ST36 and BL60 on the contralateral side to optimize the selection of acupoints. The former aims to prevent pain transition by reducing mast cell accumulation, whereas the latter produces an analgesic effect *via* supraspinal mechanisms. Although the effect of this new combination of acupoints was less potent in alleviating acute hyperalgesia than the bilateral use of ST36 and BL60, it simultaneously addressed acute pain and prevented pain transition. Our results demonstrate that EA is a promising therapeutic intervention for pain relief, with the potential for further optimization of acupoint combinations for increased preventive efficacy. Clinically, two acupoints are often selected to form a group of acupoints by EA stimulation. The combination of two acupoints not only strengthens the original effect but also has a therapeutic effect that cannot be produced by using them alone. All of the above results indicate that selection of EA acupoint combinations should be considered in clinical practice. Here, we advise that acupoints near the injured tissue can be combined with acupoints on the meridian to prevent the pain transition. Furthermore, the acupoints located on the contralateral meridian can be combined and used to alleviate acute and chronic pain.

Limitation

This study aimed to optimize the acupoint combinations for effective pain transition prevention using EA. In addition to changing the acupoint combination, other EA parameter may play a significant role in regulating mast cell accumulation and pain transition. For example, intermittent wave EA may be more effective than alternative wave EA in regulating mast cell accumulation because the waveform of EA primarily affects muscle tissue. Acupuncture-induced mast cell degranulation is believed to occur by stimulating muscle fiber contraction. Furthermore, it is necessary to further optimize the current intensity of EA on ST42 because the intensity used in this study was too high for ST42. Here, we must underscore that there are several differences between clinical practice and basic research on EA. Further clinical research is required to optimize the combination of acupoints used in EA to prevent pain transition. Our expectation is that through future EA parameter optimization studies, this technique can be more efficient in preventing pain transition and alleviating acute pain.

Conclusion

Mast cells play a key role in both HP and transition from acute to chronic pain. EA not only regulates the transition from acute to chronic pain but also prevents it by decreasing the number of mast cells in the local tissue.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Conceptualization: Junying Du, Junfan Fang, Xiaomin Jin; data curation: Junfan Fang; funding acquisition: Junfan Fang, Jianqiao Fang, and Junying Du; investigation: Junhui Ren, Na Li, Naixuan Wei, Danning Xi, Xiaomei Shao, Yi Liang, and Boyi Liu; methodology: Junhui Ren, Na Li, and Danning Xi; writing – original draft: Junying Du, Junfan Fang; writing – review & editing: Junfan Fang.

Ethical approval of studies and informed consent

This study was guided by the Regulations of the People's Republic of China on the Administration of Experimental

Animals and approved by the Ethics Committee of Animal Center affiliated with Zhejiang Chinese Medical University (Approval No. IACUC-20180319-12).

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Data availability

Data and materials are available upon request to the corresponding author.

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