Electroacupuncture Inhibits the Interaction between Peripheral TRPV1 and P2X3 in Rats with Different Pathological Pain

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Summary

Chronic pain is regarded to be one of the common and refractory diseases to cure in the clinic. One hundred Hz electroacupuncture (EA) is commonly used for inflammatory pain and 2 Hz for neuropathic pain possibly by modulating the transient receptor potential vanilloid subtype 1 (TRPV1) or the purinergic P2X3 related pathways. To clarify the mechanism of EA under various conditions of pathological pain, rats received a subcutaneous administration of complete Freund's adjuvant (CFA) for inflammatory pain and spared nerve injury (SNI) for neuropathic pain. The EA was performed at the bilateral ST36 and BL60 1 d after CFA or SNI being successfully established for 3 consecutive days. The mechanical hyperalgesia test was measured at baseline, 1 d after model establishment, 1 d and 3 d after EA. The co-expression changes, co-immunoprecipitation of TRPV1 and P2X3, and spontaneous pain behaviors (SPB) test were performed 3 d after EA stimulation. One hundred Hz EA or 2Hz EA stimulation could effectively down-regulate the hyperalgesia of CFA or SNI rats. The increased co-expression ratio between TRPV1 and P2X3 at the dorsal root ganglion (DRG) in two types of pain could be reduced by 100Hz or 2Hz EA intervention. While 100Hz or 2Hz EA was not able to eliminate the direct physical interaction between TRPV1 and P2X3. Moreover, EA could significantly inhibit the SPB induced by the co-activation of peripheral TRPV1 and P2X3. All results indicated that EA could significantly reduce the hyperalgesia and the SPB, which was partly related to inhibiting the co-expression and indirect interaction between peripheral TRPV1 and P2X3.

Key words

Electroacupuncture • Interaction • TRPV1 • P2X3 • Chronic inflammatory pain • Chronic neuropathic pain

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Introduction

Chronic pain is regularly considered to be one of the common and difficult diseases to treat in the clinic. Chronic pain includes two main types, namely chronic inflammatory pain (CIP) and chronic neuropathic pain (CNPP). Epidemiological studies have shown that there are approximately 20 % of adult people are suffering from chronic pain, and a high incidence in low-income countries (Geneen *et al.* 2017, Rabbi *et al.* 2018). In recent decades, chronic pain has led to an enormous economic burden around the world. The direct and indirect annual economic losses due to chronic pain total more than 560 billion dollars, which is significantly more than other systemic diseases (Gaskin *et al.* 2012). Clinically, chronic pain is mainly controlled by the long-term use of drugs. However, the long-term medication is often accompanied by various adverse effects, such as

gastrointestinal dysfunction, respiratory depression, drug addiction, chronic toxicity, all of which severely restrict the application of these drugs for chronic pain. Electroacupuncture (EA), which is currently recognized as a nonpharmacologic treatment with low side effects, has significant effects in treating various types of pain and pain-related diseases. However, the specific mechanism of EA is far from being elucidated.

Peripheral sensitization is one of the critical components during pain formation, in which multiple ion channels (receptors) are involved. It is known to us that the subtype 1 of transient receptor potential vanilloid family (TRPV1) and the purinergic P2X3 receptors are two of the most important receptors, and they are widely expressed in the peripheral sense-conducting pathway (Kalynovska *et al.* 2017, Levine *et al.* 2007, Spicarova *et al.* 2014, Tian *et al.* 2018, Xu *et al.* 2012). Accumulated studies have indicated that TRPV1 and P2X3 could participate in the occurrence and development of different pathological pain by different modes of activation and expression, and both of them may be potential therapeutic targets (Jung *et al.* 2017, Palazzo *et al.* 2009, Vilceanu *et al.* 2010). Previous studies have shown that there is spatial co-expression between TRPV1 and P2X3 (Chaban 2008). At the peripheral nerve terminal, the mild pain produced by the activation of the P2X3 receptor in the forearm skin of healthy volunteers could be exacerbated after exposure to intradermal injection of capsaicin or ultraviolet light (Hamilton *et al.* 2000). Thus, an amplification cascade may be elicited when TRPV1 and P2X3 are mutually promoting. It is also of great clinical significance to concentrate on blocking the interaction between them.

EA, a method of stimulating corresponding acupoints with adequate and continuous electric current, which is well accepted around the world due to its analgesic effect (Chen *et al.* 2019, Du *et al.* 2020, He *et al.* 2017). As is reported in clinical studies, the efficacy of EA at different frequencies in treating different chronic pain is still controversial, currently. Our previous studies screened frequencies of EA, and the results indicated that EA at 100 Hz for CIP (Fang *et al.* 2018) or 2 Hz for CNPP (Xia *et al.* 2019) exhibited a better analgesic effect than EA with the frequency of 2 Hz, 2/100 Hz or 100 Hz, 2/100 Hz. Extensive researches suggested that EA could achieve an analgesic effect by modulating TRPV1 and P2X3 under different pathological conditions (Chen *et al.* 2012, Fang *et al.* 2018, Jiang *et al.* 2013). At present, the exploration of the EA analgesia mechanism focuses on various pain-related molecular mechanisms and pathways, while the impact of EA on the interaction of TRPV1 and P2X3 has not yet been reported.

This research is to observe the changes in pain threshold, immunofluorescence assay, coimmuno-precipitation (co-IP), and spontaneous pain behavior (SPB) after establishing different pathological pain models and conform the intervention of EA on the interaction of TRPV1 and P2X3 under different pathological pain.

Materials and methods

Animals

One hundred and eighty-seven male Sprague–Dawley rats at age of 5 weeks (starting weight ranged from 160 to 180 g) were ordered from the Shanghai Laboratory Animal Center. Animals were housed in cages with temperature and light controlled (25 ± 2 °C, 55 % ± 5 %, 12 hours light to dark cycle) and accommodated at least 1 week to the housing. Everything we did was to minimize the rodents' number and suffering in this study. The procedures of the research were accessed and permitted by the Animal Ethics Committee of Zhejiang Chinese Medical University (ZSLL-2015-022).

Models' preparation and Grouping design

(1) Model establishment of the CIP and CNPP. CIP: The right hind paws of rats were subcutaneously administered with complete Freund's adjuvant (CFA) (100μl per rat) (Liu *et al.* 2018)and the success rate of this model was approaching to 100 %; CNPP: The rats have fasted for 12 hours before modeling, and then anesthesia was induced by isoflurane. The tibial nerve and common peroneal nerve were ligated and then cut off, whereas the sural nerve integrity was preserved without excessive traction and injury to establish a CNPP model of spared nerve injury (SNI) (Decosterd *et al.* 2000). Due to the difficulties of SNI operating, the success rate of SNI was up to approximately 70 %.

(2) Experimental design. There were two parts of this research that were designed. In experiment I, the rodents were stochastically divided into four groups (n=6), the control, the CFA, the sham EA, the 100 Hz EA for CIP, and the sham SNI, the SNI, the sham EA, the 2 Hz EA for CNPP, respectively. In experiment II, the rodents were stochastically divided into 2 groups (n=6) in

each model of pain, the capsaicin + α, β-meATP, the 100Hz EA + capsaicin + α, β-meATP for CIP; the capsaicin + α, β-meATP, the 2Hz EA + capsaicin + α, β-meATP for CNPP. The rats were firstly subcutaneously injected with capsaicin into plantar, and α, β-meATP injection was conducted in 1 min.

EA administration

The EA administration was conducted by following the steps in previous studies (Fang *et al.* 2018, Liu *et al.* 2018). The rodents were placed in customized cotton cases (Patent No. ZL201420473579.9). Bilateral acupoints ST 36 and BL 60 were chosen and sterile needles were punctured into the skin at a depth of 5 mm. Two pairs of acupoints were stimulated with different frequencies (100 Hz for CFA and 2Hz for SNI) by HANS Acupoint Nerve Stimulator (HANS - 200A, Huawei Co., Ltd., China). The intensity of EA was ranging from 1mA to 2mA, a total of 30 min for once each day. The sham EA was needled subcutaneously at ST36 and BL 60 at a depth of approximately 1 mm and the needles were linked to the electrodes without electric current passes by.

Preparation and injection of drugs

Drugs in this study were α, β-methyle-neadenosine 5'-triphosphate lithium salt (α, β-meATP), a P2X3 agonist (Sigma-Aldrich Co., Ltd) and capsaicin, a TRPV1 agonist (Sigma-Aldrich Co., Ltd). The capsaicin was dissolved into a 100 % DMSO, 50 mg/ml stock solution and was diluted with the solution (5 % Tween 80 saline solution) before injection. The α, β-meATP was dissolved into sterile 0.9 % NaCl, 100 mg/ml stock solution, and deliquated with precooled sterile saline before injection. The volume of drug injections was ensured to be 50 μl and was delivered into the on the dorsum of the hind paw with a small diam needle at 30G (Sawynok *et al.* 2006). The α, β-meATP was injected in 1 min after administration of the capsaicin.

Behavioral tests

(1) Mechanical hyperalgesia testing. The mechanical paw withdrawal thresholds (mPWTs), that suggested mechanical hyperalgesia, was observed according to papers described previously (Chaplan *et al.* 1994). The mPWTs were observed before modeling, 1 d after modeling but before EA stimulation, 1 d after EA administration, and 3 d after EA administration. The mPWTs were observed between 9: 00 and 16: 00. The rats were accommodated in transparent observation chambers for 30 min once a day for 3 days. During the observation, rats were allowed to calm down for approximately 20 min. The different thickness of von Frey hairs that were made of nylon monofilaments was inserted perpendicularly onto the center of the hind paw (CFA) or the sural innervated hind paw surface (SNI), then held for approximate 6–8 s to conform mPWTs in serial order (0.4, 0.6, 1, 2, 4, 6, 8, 15, and 26 g). The 4 g hair was first used. When a withdrawal response of the paw to the hair did not appear, a mark "O" was written down and the next stronger force hair was applied. When a paw withdrawal was performed, an "X" was recorded immediately and the next weaker force hair was chosen. Six response data points would be collected during the testing. Counting of these 6 responses recorded would not start until the first encounter of "O" and "X" appeared. To avoid continuous positive or negative responses, values of 0.4 g and 26 g were decided to be the minimum and maximum of PWTs. The mPWTs were calculated by formula as follows: $(10^{[Xf + \kappa\delta]})/10000$, in which Xf represents the value of the ultimate hair applied, κ means the tabular value for the models of positive/negative responses and δ expressed as the mean differences between different stimulus (0.231 is determined here).

(2) Spontaneous pain behaviors test. The rodents were adapted to Plexiglas observation chambers for approximately 30 min for 3 consecutive days. After models were successfully established, the rats were put in the chambers for 5 min and then the drugs were injected subcutaneously into the dorsum of the hind paws according to the experimental protocol. The rats were then placed back into the individual chamber. Spontaneous flinching times engaged that were related to paw lifting or shaking of the hind paw were counted every 2 min during a 20 min observation. Spontaneous flinching times caused by adapting to the environment should be excluded. SPB was counted and recorded by two different individuals who handled the injections.

Immunofluorescence assay

After rats were anesthetized, they were perfused with pre-cooled saline (4 °C) and 4 % paraformaldehyde through the ascending aorta successively. Ipsilateral DRG of the L4-L6 was quickly removed, soaked in 4 % paraformaldehyde for 3 hours, dehydrated with 15 %, and 30 % sucrose solution, immediately irrigated with liquid nitrogen for further steps. After being embedded with an optimal cutting temperature compound (SAKURA,

USA) and frozen, then the tissues were cut into slices of 14 μm thick. All slices were infiltrated in Tris-buffered saline (TBST) containing 5 % donkey serum and sealed under 37 °C for 1 h. Next, slices were covered by a solution of the TRPV1 antibody (1: 1000, Abcam Co., Ltd, USA) and P2X3 antibody (1: 400, Santa Cruz Co., Ltd, USA) in TBST with 5 % donkey serum and then incubated whole night through at 4 °C. Then, the slices were immersed in the secondary antibody of FITC (1: 1000, Abcam Co., Ltd., USA) and Alex-647 (1: 400, Jackson Lab, USA) at 37 °C for 1 h (free from light). Images were immediately shot by an inverted laser confocal microscope (A1R, Nikon Co., Ltd, Japan) with a 10-fold objective lens. Both positive cells of TRPV1 or P2X3 and co-expressed TRPV1 and P2X3 were counted through Image-Pro Plus 6.0. Then, 3-5 rats were selected from each group, and each rat had 5 sections randomly selected for counting. Expression ratio=co-express number of TRPV1 or P2X3 / total number of positive cells (TRPV1 + P2X3) * 100 %.

Co-immunoprecipitation

As described previously (Hall 2004), the rats were abdominally anesthetized, and then ipsilateral L4-L6 DRGs were quickly harvested. The samples were weighed and put into RIPA (100 mM PMSF added before use) according to proper proportions (1: 30). The samples were homogenized and put in an ice bath for 15 min. After being centrifugated, the supernatant was transferred into a new tube. A part of the total protein was taken as an input after the protein concentration was quantitated by the BCA kit (Beyotime Bio Co., Ltd, China). The remaining 800 mg of protein were taken for protein precipitation. The TRPV1 antibody (3 μl, Abcam, USA) or P2X3 antibody (5 μl, Santa Cruz., USA) was added to 800 mg of total protein. The mixture of antigen and antibody was slowly shaken overnight at 4 °C. Then, 40 μl suspended protein A/G plus-agarose was added into the protein solution to precipitate it. Similarly, the mixture was slowly shaken at 4 °C for 4 h. The complexes of agarose beads antigen and antibody were collected by centrifugation for 5 min at 3000 g after being washed with precooled PBS. The beads were boiled in 2× loading buffer at 100 °C for 5 min to dissociate antigens, antibodies, and beads, and the supernatant was cautiously aspirated. Next, the supernatant was separated by electrophoresis and then transferred onto a membrane of polyvinylidene fluoride (PVDF). After blocked by 5 % (w/v) skim milk TBST for 1 h under room temperature (RT), the membrane was immersed in a diluted solution of TBST with P2X3 antibody (1: 1000, Santa Cruz Co., Ltd, USA) or TRPV1 antibody (1: 1000, Abcam Co., Ltd, USA) through the whole night at 4 °C. Then, the membrane was infiltrated with the secondary antibody (1: 1000, Abcam Co., Ltd, USA) that was conjugated with horseradish peroxidase (HRP) at RT for 2 h. Solution A and B from the WESTAR SUPERNOVA or the BeyoECL Plus luminescent kit were mixed equally. The mixed solution was put on the membrane and allowed to react for 2 min at RT until being photographed by ImageQuant LAS 4000 (GE, USA).

Statistical analysis

The data were performed by $\bar{\chi}\pm$ SEM (means ± standard error). The data of mPWTs were analyzed using repeated-measures ANOVA. Differences of groups at different time points were measured by a one-way ANOVA for independent samples. When the variance homogeneity appears, the least significant difference (LSD) testing was selected for multiple comparisons; when the variance heterogeneity appears, the Dunnett's T3 test was chosen. An independent T-test was selected to analyze the differential of the two groups. $P<0.05$ was regarded to be the standard of obvious significance.

Results

The intervention of EA on different pathological pain

To investigate the analgesic effect of EA on mPWTs in rats under different pathological pain, we established the CIP induced by CFA and CNPP induced by SNI, and then the mPWTs were observed and EA was delivered in specific timing (Fig.1A). After injection, contrasted with the control, the mPWTs of the CFA reduced obviously ($P<0.01$) which implied that the CIP model was successfully established. While the 100Hz EA, but not the sham EA, up-regulated the mPWTs significantly 1 d and 3d ($P<0.01$, $P<0.01$) in contrast with the CFA (Fig. 1B). After the selective nerve injury, the ipsilateral mPWTs of the SNI decreased significantly ($P<0.01$), which implied that the model of CNPP had been established. When compared with the SNI, the mPWTs of the 2Hz EA, not the sham EA, increased significantly on the third day since EA treatment ($P<0.05$) (Fig. 1C). Therefore, the above results indicated that hyperalgesia which is induced by the model of CFA or SNI can be inhibited significantly by 100Hz or 2Hz EA.

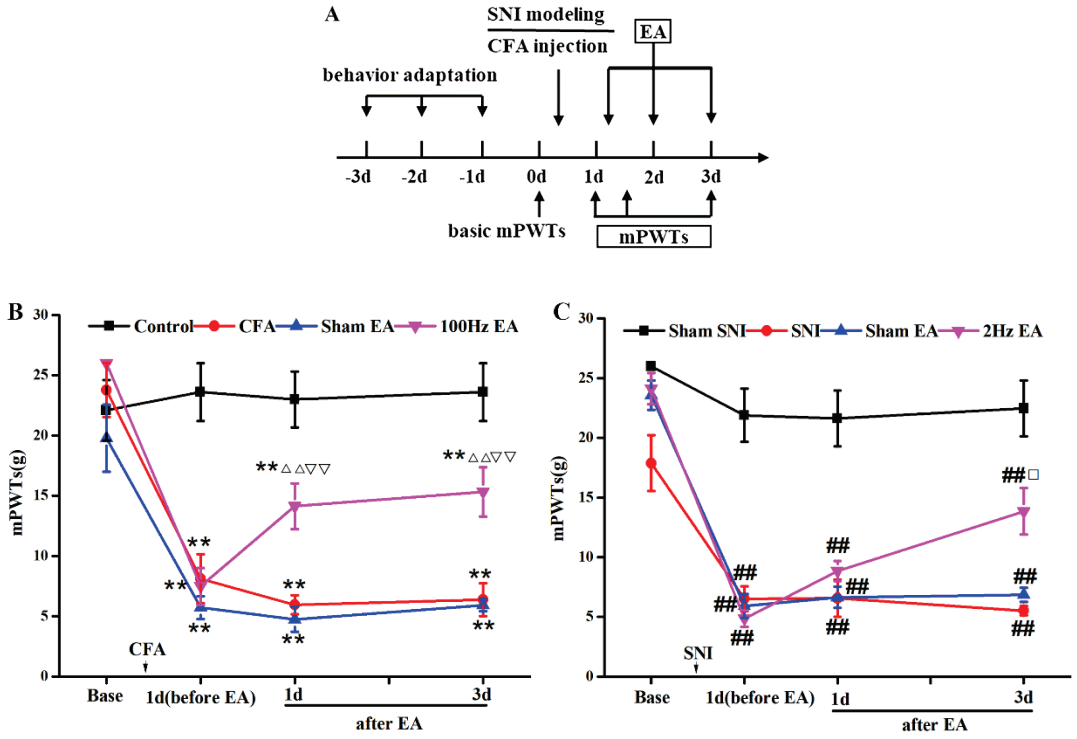

Fig. 1. The intervention of EA on rats with CFA and SNI. (**A**) The procedure of experiments. (**B**) The analgesic effect of EA (100Hz) on the mPWTs of rats with CFA. (**C**) The analgesic effect of EA (2Hz) on the mPWTs of rats with SNI. Data were performed as $\bar{\chi}\pm$ SEM, n=6. Contrasted with the control, ** P<0.01; contrasted with the CFA, △△P<0.01; contrasted with the sham EA, ▽▽P<0.01; contrasted with the sham SNI, ##P<0.01; contrasted with the SNI, □P<0.05.

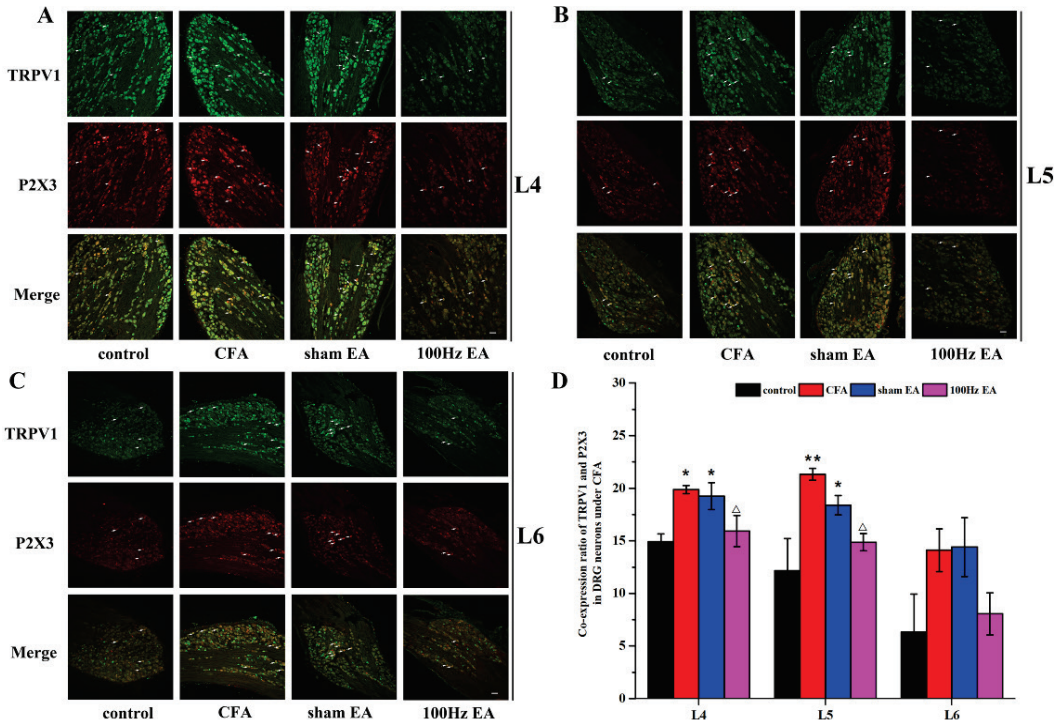

Fig. 2. The intervention of EA (100Hz) on the co-expression ratio of TRPV1 and P2X3 in ipsilateral DRG neurons of rats with CFA. (**A-C**) Immunofluorescence images of DRGs at L4-L6 in the groups of control, CFA, sham EA, 100Hz EA. Sections of green marks were for neurons of TRPV1, red marks were for P2X3-neurons and yellow labeling was for co-expression of TRPV1 and P2X3, and parts of positive neurons were pointed out with white arrows. Scale bars=100 μm. (**D**) Changes of co-expression ratio between TRPV and P2X3 at different segments of ipsilateral DRG in CFA rats. Data were shown as $\bar{\chi}\pm$ SEM, n=6. Contrasted with the control, *P<0.05, **P<0.01; contrasted with the CFA, △P 0.05.

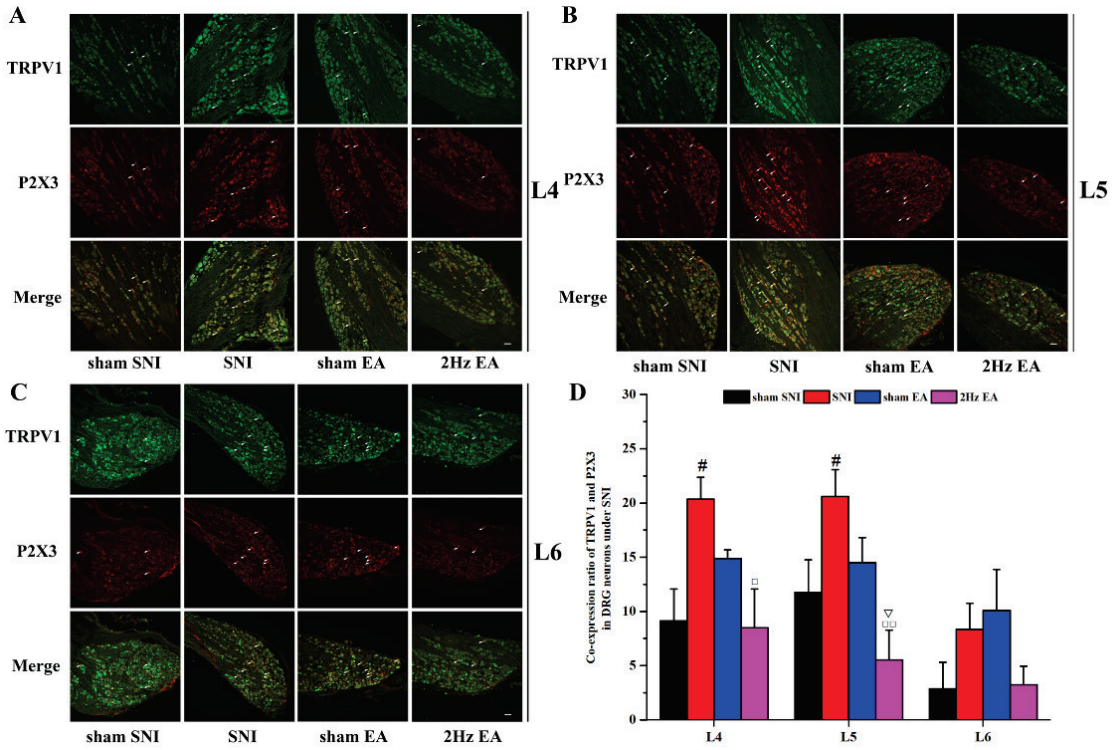

Fig. 3. The intervention of EA (2Hz) on the co-expression ratio of TRPV1 and P2X3 in ipsilateral DRG neurons. (**A-C**) Immunofluorescence images of DRGs at L4-L6 in the groups of sham SNI, SNI, sham EA, 2Hz EA. Sections of green marks were for neurons of TRPV1, red marks were for P2X3-neurons and yellow labeling was for co-expression of TRPV1 and P2X3, and parts of positive neurons were pointed out with white arrows. Scale bars=100 μm. (**D**) Changes of the co-expression ratio of TRPV1 and P2X3 at different segments of ipsilateral DRG in SNI rats. Data were given as $\bar{\chi}\pm$ SEM, n=6. Contrasted with the sham SNI, #P<0.05; contrasted with the SNI, □P<0.05, □□P<0.01; contrasted with the sham EA, ▽P<0.05.

The EA reduced the increased co-expression of TRPV1 and P2X3 in ipsilateral L4-L6 DRG neurons

We then observed the intervention of EA (100Hz) on the co-expression ratio of TRPV1 and P2X3 in small-medium DRG neurons of CFA rats (Fig. 2). Contrasted with the control, the co-expression ratio of TRPV1 and P2X3 in the CFA increased in DRGs at L4 and L5 (P<0.05 and P<0.01), but not at L6 (P>0.05). Contrasted with the CFA and the sham EA, the co-expression ratio of TRPV1 and P2X3 in the 100Hz EA was decreased by EA (100Hz) stimulation in the L4 and L5 (P<0.05 and P<0.05) DRG, but not in the L6 (P>0.05). Though the change of co-expression ratio between TRPV1 and P2X3 in L6 DRG showed no significance among the four groups, the trend of the change among them was similar to that in the L4 and the L5 DRG. No significant difference showed up in the CFA in contrast with the sham EA. We also observed the intervention of EA (2Hz) on the co-expression ratio of TRPV1 and P2X3 in small-medium DRG neurons of SNI rats (Fig. 3). Contrasted with the sham SNI, the co-expression ratio of TRPV1 and P2X3 in the SNI remarkably increased in the L4 and the L5 (P<0.05 and P<0.05) DRG, but not in the L6 (P>0.05). Contrasted with the SNI, EA (2Hz) could significantly inhibit the co-expression ratio of TRPV1 and P2X3 in the L4 and the L5 (P<0.05 and P<0.01) DRG, but not in the L6 (P>0.05). What's more, the co-expression ratio of the 2Hz EA in the L5 DRG was significantly lower than the sham EA. Similarly, none of the significant variations among the four groups in the L6 DRG was observed, but the trend of change among them was similar to that in the L4 and the L5 DRG.

Co-immunoprecipitation of TRPV1 and P2X3 in ipsilateral DRG neurons

This part of the experiment was designed to observe the regulation of EA on the physical interaction between TRPV1 and P2X3 in CFA and SNI rats. As we could see in the CFA rats, the input lanes pointed out the accurate location of TRPV1 or P2X3 bands. The outcomes in CFA rats indicated that the visible signals between TRPV1 and P2X3 existed in ipsilateral DRG of the control, the CFA and the sham EA, both under condition of TRPV1 precipitation, P2X3 blotting and in P2X3 precipitation, TRPV1 blotting; however, obvious

signals in the 100Hz EA still appeared (Fig. 4A). In the SNI rats, the lanes of input showed the accurate location of TRPV1 or P2X3 bands and similar results were also observed (Fig. 4B). All of the results suggested that the interaction between TRPV1 and P2X3 is relatively stable, which cannot be eliminated after EA (100Hz or 2Hz) intervention under different pain.

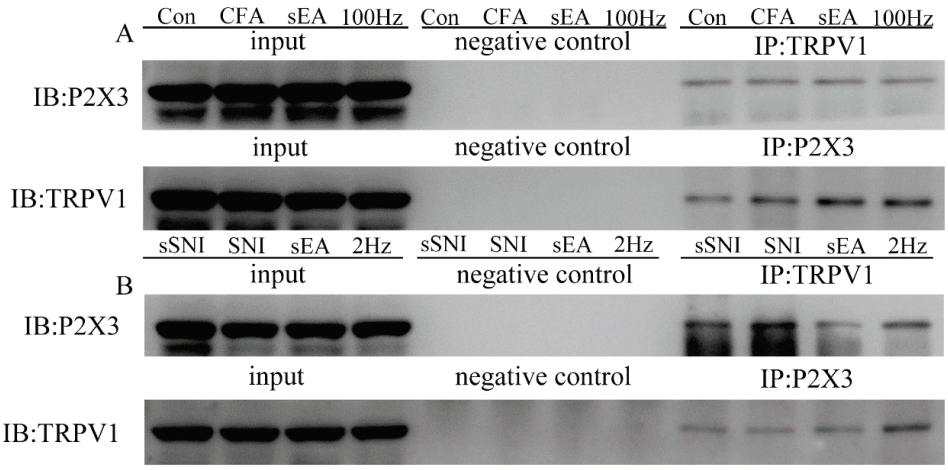

Fig. 4. The effect of 100Hz EA and 2Hz EA on the physical interaction of TRPV1 and P2X3 in rats DRGs with CFA and SNI. (**A**) In CFA, the precipitated protein was TRPV1 and the blotted protein was P2X3 (up-side), the precipitated protein was P2X3 and the blotted protein was TRPV1 (down-side). (**B**) In SNI, the precipitated protein was TRPV1 and the blotted protein was P2X3 (up-side), the precipitated protein was P2X3 and the blotted protein was TRPV1 (down-side). The sSNI and the sEA were the abbreviations of sham EA and sham SNI.

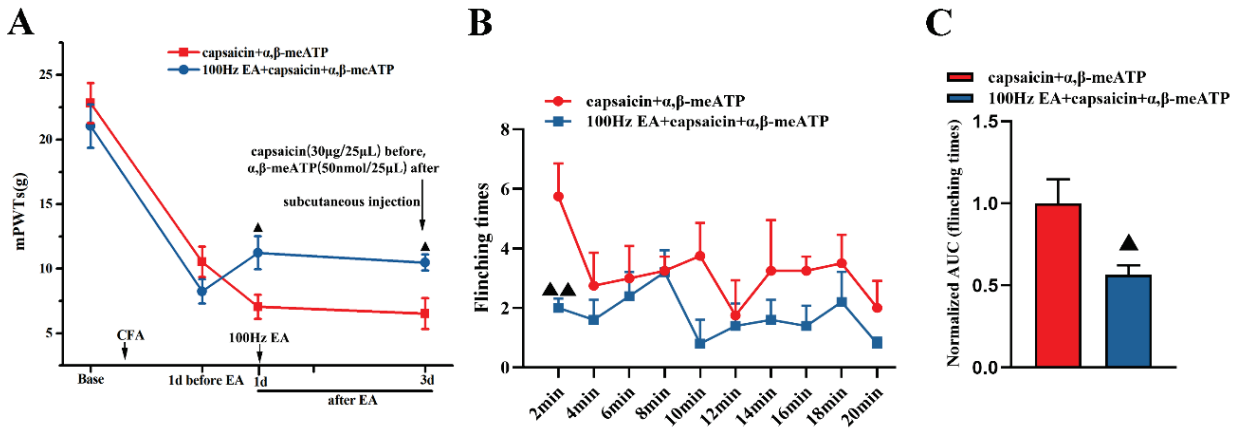

Fig. 5. The effect of 100Hz EA is tested on the co-activation of TRPV1 and P2X3 receptors after injection of CFA. (**A**) The mPWTs observation of 100Hz EA intervention on the co-activation between TRPV1 and P2X3 in CFA rats. (**B**) The intervention of EA (100Hz) on the spontaneous flinching times at every 2 min. (**C**) The columns of normalized AUC of total flinching times within 20 min of two groups. Data were presented as $\bar{\chi}\pm$ SEM, n=6. Contrasted with the capsaicin + α, β-meATP, ▲P<0.05, ▲▲P<0.01.

The 100Hz EA inhibited the SPB induced by the co-activation between TRPV1 and P2X3 at the peripheral nerve terminal of CIP rats

To explore the intervention of EA (100Hz) on the co-activation between TRPV1 and P2X3, this experiment observed the changes of SPB in CFA rats. The observation was the intervention of 100Hz EA on the SPB induced by the co-activation interaction of peripheral P2X3 and TRPV1. Compared with the capsaicin + α, β-meATP, EA increased the mPWTs of the 100Hz EA + capsaicin + α, β-meATP significantly at 1 d and 3 d (P<0.05 and P<0.05) after EA treatment (Fig. 5A). The results showed that the number of flinching times in the 100Hz EA + capsaicin + α, β-meATP was significantly reduced in contrast with the capsaicin + α, β-meATP at 2 min after drugs injection (P<0.01) (Fig. 5B). Besides, the normalized AUC (area under the curve) of total flinching times in the 100Hz EA

+ capsaicin + α, β-meATP was lower than the capsaicin + α, β-meATP (P<0.05) during 20 min after injection (Fig. 5C).

The 2Hz EA inhibited the SPB induced by the co-activation between TRPV1 and P2X3 at the peripheral nerve terminal of rats with CNPP

Meanwhile, we also observed the 2Hz EA intervention on the co-activation between TRPV1 and P2X3 through SPB in SNI rats. The mPWTs of the 2Hz EA + capsaicin + α, β-meATP increased significantly after 3 day's EA treatment in contrast with the capsaicin + α, β-meATP (P<0.05) (Fig. 6A). The number of flinching times in the 2Hz EA + capsaicin + α, β-meATP was significantly decreased when compared with the capsaicin + α, β-meATP at 2 min, 6 min, and 16 min (P<0.05, P<0.05 and P<0.01) after drug injection (Fig. 6B). The results also showed that EA reduced the normalized UAC of total flinching times in the 2Hz EA + capsaicin + α, β-meATP significantly when compared with the capsaicin + α, β-meATP (P<0.05) during observation for 20 min (Fig. 6C).

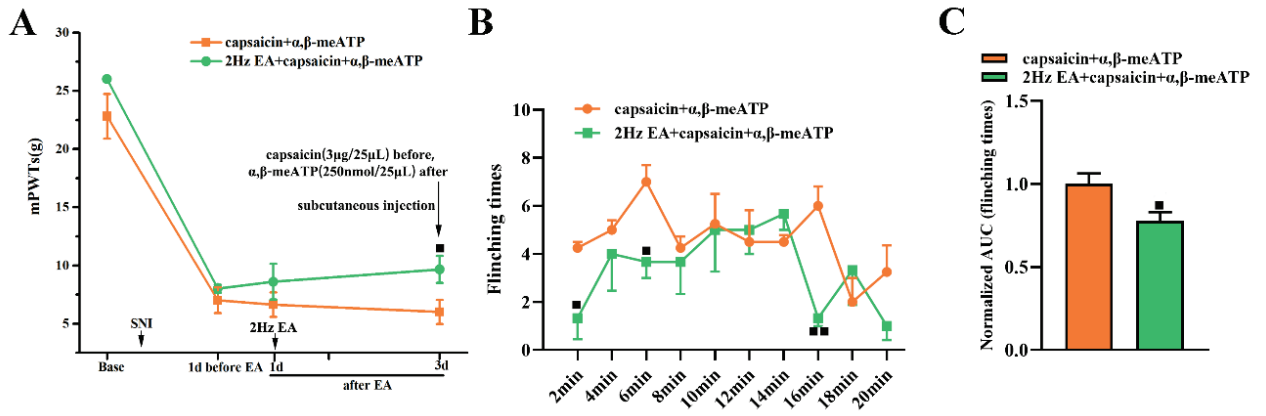

Fig. 6. The effect of 2Hz EA was tested on the co-activation of TRPV1 and P2X3 receptors after SNI modeling. (**A**) The mPWTs observation of EA (2Hz) intervention on the co-activation between TRPV1 and P2X3 in SNI rats. (**B**) The intervention of EA (2Hz) on the spontaneous flinching times at every 2 min. (**C**) The columns of total flinching times within 20 min of two groups. Data were performed as $\bar{\chi}$± SEM, n=6. Contrasted with the capsaicin + α, β-meATP, ■ P<0.05, ■■P<0.05.

Discussion

Chronic pain is regarded to be a common disease. The optimization of its diagnosis and treatment and the exploration of relevant mechanisms have been a topic of interest in the field of pain research. Previous studies reported that 1 d after CFA injection, the ipsilateral hyperalgesia increased significantly, which reached its peak on the seventh day, and then reached the plateau stage and sustained for 4-8 weeks (Tekieh *et al.* 2011). Another study found that 1 d after SNI, the ipsilateral pain threshold significantly decreased when compared with the healthy controls and maintained for at least 2 weeks (Richner *et al.* 2011). Our results showed that 1 d after modeling of CFA or SNI, the ipsilateral hyperalgesia in the models of both CFA and SNI increased significantly, which suggested that the models were established successfully. Consistent with previous studies (Richner *et al.* 2011), the ipsilateral pain threshold was maintained at a low level of 1 d to 3 d after the establishment of the models.

The antinociceptive effect of EA has already been well accepted. Accumulated clinical and basic researches reported that the pain relief of EA could be altered following changes in EA parameters (Fang *et al.* 2018, Smith *et al.* 2011). The frequency of EA stimulation on acupoints is one of the most concerned and controllable parameters, which makes antinociceptive regulation of EA different under CIP and CNPP. Jiang and his colleagues (Jiang *et al.* 2001) reported that compared with 2 Hz EA, 100 Hz EA could perform a better antinociceptive effect when transcutaneous electrical acupoint stimulates bilateral ST36 of rats with CIP. Another study also showed similar results (Fang *et al.* 2018, Xiang *et al.* 2019). When EA with frequencies of 2 Hz, 15 Hz, and 100 Hz were used to stimulate the bilateral ST36 and Yanglingquan (GB34) of rats with spinal nerve ligation, the suppression of EA (2 Hz) on hyperalgesia was greater when contrasted with that of EA with 15 Hz and 100 Hz (Shou *et al.* 2017). In

the present results, EA (100 Hz) was used to treat CFA rats and EA (2 Hz) was used to treat SNI rats in this research. Our results indicated that EA (100 Hz) could inhibit ipsilateral hyperalgesia of rats after 1 d of EA treatment, and this effect could be maintained until 3 d after the CFA injection. Under the condition of CNPP, EA (2 Hz) could also obviously suppress the ipsilateral hyperalgesia till 3 day's EA treatment at least.

Both TRPV1 and P2X3 receptors occupied important positions in the initiation and development of pain, and they co-expressed with each other in the neurons of both DRG and TG (North 2004, Xiao *et al.* 2015). Therefore, there may be compact interaction of function between TRPV1 and P2X3 receptors. In the study of CIP in the intestine, Kiyatkin and his colleagues found that when the nociceptive sensitization of intestinal muscles and mucosa decreased in TRPV1 or P2X3 receptor knock-out mice, and the hyper-sensation of the afferent nerve of the large intestine was inhibited when antagonist of TRPV1 or P2X3 was administered (Kiyatkin *et al.* 2013). Thus, it suggests that there is a synergistic effect between TRPV1 and P2X3 in rats with CIP. In CNPP, Saloman and his colleagues (Saloman *et al.* 2013) found that when the trigeminal neuralgia rats were pretreated with TRPV1 antagonist AMG9810 on masseter muscle, mechanical hyperalgesia of which was inhibited following the injection of P2X3 agonist α, β-meATP. Additionally, they observed that the transient current of Ca^{2+} induced by capsaicin increased after rats pretreated with the P2X3 agonist. These results indicate that there may be a synergistic relationship between peripheral TRPV1 and P2X3 in CNPP induced by trigeminal neuralgia. In this study, we investigated that the co-expression ratio of TRPV1 and P2X3 robustly increased when different pathological models were successfully established, which was consistent with the researches described previously.

Accumulated studies have reported that both TRPV1 and P2X3 can be inhibited by EA stimulation under different pathological pain. TRPV1, S100-B protein, opioid peptides, and adenosine were down-regulated by the stimulation of EA on ST36 in mice to inhibit Nav1.8 and thereby decreased inflammatory hyperalgesia (Liao *et al.* 2017). Also, some researchers found (Lee *et al.* 2012) that low frequency (frequency under 5Hz) EA could up-regulate the decreased mPWTs induced by SNI through reducing TRPV1 expression in ipsilateral undamaged DRG in rats. In terms of EA intervention on P2X3, the latest study observed that down-regulation of EA (2 Hz) on the over expressions of P2X3 and CGRP receptors in DRGs could effectively inhibit the hyperalgesia induced by type 2 diabetic neuropathic pain (He *et al.* 2017). Visceral pain could also be attenuated by EA (100 Hz), which was associated with the reversal of the upregulation of TRPV1 in adult rats (Zhu *et al.* 2016). Therefore, to explore the mechanisms underlying EA for its satisfied analgesic effects on different pathological pain, we detected the co-expression ratio of TRPV1 and P2X3 in ipsilateral DRG. The result suggested that under different pathological pain, EA (100Hz or 2Hz) could down-regulate the elevated co-expression of TRPV1 and P2X3 in L4 and L5 DRG, but not in L6, under the condition of CIP or CNPP. This result indicated that the analgesic effect of EA was partly through inhibiting the co-expression of TRPV1 and P2X3; nevertheless, EA might also regulate TRPV1 and P2X3 respectively to alleviate pain as previously studies described (Fang *et al.* 2018, Liao *et al.* 2017, Lin *et al.* 2015, Yang *et al.* 2018, Yen *et al.* 2019).

Accumulated evidences show that there are direct physical and indirect interactions between TRPV1 and P2X3 in vivo. A physiological study (Stanchev *et al.* 2009) had observed that the cross-talks between TRPV1 and P2X3 in DRG neurons were blocked by truncating Glu362 at the C-terminal of P2X3 receptor. Besides, when TRPV1 receptors were activated in CIP and CNPP, it could strengthened the release of SP, CGRP, and other neurotransmitters (Kang *et al.* 2020, Magnussen *et al.* 2015) and activated protease and tryptase secreted by mast cells (Vincent *et al.* 2015), thereby activated TRPV1 in return (Du *et al.* 2019). When these neurotransmitters upregulated the sensitivity of TRPV1, they could also increase the activation of P2X3 receptor in vivo (Jung *et al.* 2017), which might be one of the mechanisms underlying the mutually reinforcing relationship between peripheral TRPV1 and P2X3 under different pathological conditions. Another study (Saloman *et al.* 2013) suggested that TRPV1 in DRG neurons of rats could be phosphorylated and activated by PKC and CaMKII, and P2X3 was related to the activation of CaMKII and PKC as well. Therefore, to further investigate the interaction of TRPV1 and P2X3, we applied the co-IP assay. As co-IP experiments in previous studies have routinely suggested that there is a direct physical interaction of TRPV1 and P2X3 in DRG neurons under physiological pain conditions (Saloman *et al.* 2013). Since co-IP can be used to detect the relatively stable interaction between two

proteins, the results above suggested that EA could not eliminate the physical interaction between TRPV1 and P2X3 in rats with different pathological pain. In terms of pain behavior, previous papers had reported that the SPB induced by overexpressed TRPV1 at the peripheral nerve terminals under different pathological conditions was enhanced by P2X3 agonists (Sawynok *et al.* 2006, Xiang *et al.* 2008). However, we found that the enhancement of TRPV1 agonists on the SPB induced by P2X3 agonists had been weakened following EA (100Hz or 2Hz) treatment for 3 days in CFA and SNI rats, which might be related to the indirect interaction between TRPV1 and P2X3.

Unfortunately, our study still presents some limitations. Although we initially investigated that EA could not thoroughly eliminate the physical interaction between TRPV1 and P2X3, whether EA could weaken or reduce the interaction remain unknown due to the absence of rats with a mutant Glu362 site of TRPV1 and P2X3 and the related antibodies at that time. Meanwhile, whereas behavioral evidence indicated the intervention of EA on the co-activation between TRPV1 and P2X3, the potential mechanism in which is still unrevealed. In conclusion, to further explore the specific mechanism of EA intervention on the direct or indirect interaction between TRPV1 and P2X3, our future research will put light on exploring interacted sites, modulators, and dynamic changes in the interaction between them.

Conclusion

The hyperalgesia of pain induced by CFA and SNI is associated with the interaction between TRPV1 and P2X3 in the DRG. One hundred Hz or 2Hz EA can significantly reduce hyperalgesia induced by CFA or SNI since 1d or 3d after treatment. This analgesic effect of both 100Hz and 2Hz EA treatment may be partly related to the inhibition on the co-expression and indirect interaction between TRPV1 and P2X3. However, EA can't thoroughly eliminate the physical interaction between TRPV1 and P2X3.

Conflict of Interest

There is no conflict of interest.

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