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Full Length Article

Electroacupuncture Alleviates Anxiety-like Behavior in Pain Aversion Rats by Attenuating the Expression of Neuropeptide Y in Anterior Cingulate Cortex

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ABSTRACT

Background: Pain is considered as a multidimensional conscious experience that includes a sensory component and a negative affective-motivational component. Electroacupuncture (EA) is widely used to treat pain and pain-induced negative emotions, however, little is known about the mechanisms underlying the effect of EA.

Objective: This study investigated the effect of EA on alleviating the anxiety-like behaviors in pain aversion rats and its anterior cingulate cortex (ACC) regulation mechanism.

Methods: After a Freund's complete adjuvant (CFA)-conditioned place aversion (C-CPA) model was established in rats, EA treatment (2/100 Hz, 30 min, once/day, 4 days totally) was applied at bilateral Zusanli (ST36) and Kunlun (BL60) acupoints. Von Frey filaments were used to measure changes of pain withdrawal threshold (PWT) at indicated time points. Elevated zero maze (EZM) was used to investigate the changes of pain-related anxiety and CPA was used to investigate the changes of pain aversion. The protein expression levels of GAD67, PV, and NPY in ACC were detected by Western blotting.

Results: Compared with the control group, the staying time in the "CFA-paired compartment" was significantly reduced, and the PWT was decreased in model group. In the EZM assessment, the distance and the time in open arm, as well as the number of open arm entries of model group were significantly lower than those in the control group. In the CPA assessment, the time spent in the "CFA-paired compartment" was significantly decreased in model group compared with control group, and EA reversed the changes in pain sensation and in pain-related emotions. Western blotting showed that the NPY level, but not the levels of GAD67 and PV, was significantly increased in the ACC of the model group compared to that of the control group. The increased expression of NPY in the ACC was significantly downregulated by EA, while sham EA produced no such effect.

Conclusion: EA can effectively relieve the pain and pain-related emotions, and its mechanism may be achieved by down-regulating the expression of NPY in the ACC.

1. Introduction

Chronic pain not only causes painful sensations, but also often causes adverse emotional reactions such as depression and anxiety (Xie et al., 2012; Williams and Craig, 2016). It is reported that more than 50% chronic pain patients suffering from anxiety disorder, which greatly increases the patients' pain burden (Feingold et al., 2017). While there have been great advances in understanding of the neuroscience basis of pain sensation, many researchers have begun to turn their attention to the mechanisms of pain emotion (Lumley et al., 2011).

The anterior cingulate cortex (ACC), an important part of the limbic system, has demonstrated the role in the processing of information related to pain and emotions (Meda et al., 2019; Bliss et al., 2016; Gomtsian et al., 2018). Clinical studies showed that ACC is activated in humans during pain and its activity is modulated during pain management (Peyron et al., 2000; Antioch et al., 2020). Preclinical studies also

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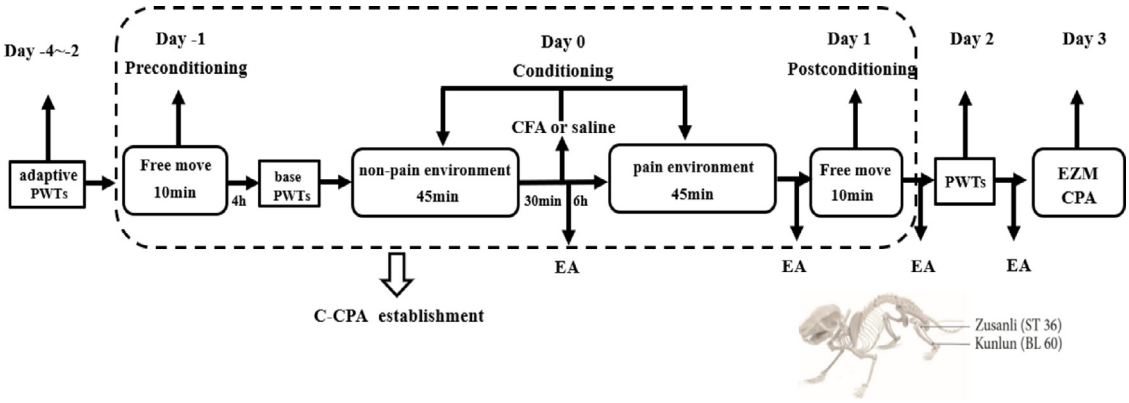

Fig. 1. Experimental flow chart.

found that ACC activation is closely related to mood disorders caused by chronic pain (Barthas et al., 2015; Jarrin et al., 2020) and ACC lesions are associated with pain aversion. For example, it has been shown in rats that ACC lesions significantly reduce the aversion produced by hind paw formalin injection (Johansen et al., 2001) and block conditional preference of analgesics in neuropathic pain (Qu et al., 2011).

GABA is the main inhibitory neurotransmitter in ACC (Kim et al., 2011). GABAergic interneurons constrain the spread of excitation by controlling generation and timing of spiking of adjacent glutamatergic pyramidal neurons (Pouille and Scanziani, 2001). It has been proposed that the hyperactivity of ACC in response to injury results from secondary loss of GABAergic inhibition function (Narita et al., 2011). In addition to their role in modulation of pain sensation, GABAergic neurons in ACC are crucial for mediating nociceptive affect-motivation and as such conferring an aversive value to the noxious stimulus (Ang et al., 2015). Indeed, a study utilizing injections of GABAA receptor agonist muscimol to silence discrete areas of limbic cortex found that interaction of ACC with prelimbic cortex is responsible for acquisition and expression of aversive dimension of pain assessed by formalin-CPA (Jiang et al., 2014). While the GABA system is clearly involved in the aversive dimension of pain, the role of specific GABAergic interneurons mediating such activity remains to be explored. GABAergic interneurons are heterogeneous and are classified into five main groups based on the expression of specific molecular markers (DeFelipe et al., 2013) of which interneurons expressing parvalbumin (PV) and neuropeptide Y(NPY) have been reported with roles in emotional regulation (Schmeltzer et al., 2016; Page et al., 2019).

Electroacupuncture (EA) has been widely used to treat pain and pain-induced negative emotions, such as anxiety, depression and cognitive impairment (Yin et al., 2020; Zheng et al., 2020; Shao et al., 2015). Our previous studies have shown that the effect of EA on chronic inflammatory pain-related emotions is closely related to its modulation of ACC neuronal activity (Du et al., 2017). The effect of EA on GABAergic inhibition in ACC has, however, not been explored in detail. In this study, we aimed to explore the role of GABAergic system in ACC under chronic pain condition, with emphasis on the PV and NPY expressing interneurons. In addition, we investigated whether and how EA therapy alleviated nociception-induced aversion and anxiety-like behaviors in adult rats.

2. Materials and Methods

2.1. Animals

Adult male healthy Sprague–Dawley (SD) rats (weighing 200 ± 20 g) were provided by the Shanghai Laboratory Animal Center, Chinese academy of sciences [SCXK (HU) 2018-0006]. During breeding, rats were group-housed with a maximum of four animals per cage (temperature: 25 ± 2°C, humidity: 40–60%), kept under a reversed 12 h light/dark cycle and given food and water *ad libitum*. The procedure received approval from the animal protection agency and the use committee (IACUC-20180319-12). All the experimental protocols were in accordance with the Regulations on the Control of Laboratory Animals of the People's Republic of China.

2.2. Experimental design

Rats were randomly assigned into four groups: (1) control group, (2) CFA group, (3) CFA + EA group, and (4) CFA + sham EA group. Pain and aversion behavior tests were performed according to the schedule (Fig. 1). On Day 0 and Day 2, measurement of paw withdrawal threshold (PWT) was carried out to detect the change of pain thresholds. On Day 1, the CFA-induced conditioned place aversion (C-CPA) model was established, and the test of CPA was repeated on Day 3 to detect the pain-induced aversion of rats. On Day 3, pain-induced anxiety emotion was observed by elevated zero maze (EZM). The protein level of ACC in rats was detected by Western blotting.

2.3. C-CPA model establishment

The C-CPA model was divided into three stages. All stages were performed in a conditional position box which was divided into two equal square (30 cm × 30 cm) compartments with a partition. The wall of compartment "A" was covered with evenly distributed white dots, and the corner of the box away from the partition was coated with cinnamon oil; The wall of compartment "B" was covered with evenly distributed white equilateral triangles, and the corner of the box away from the partition was coated with 5% acetic acid. Thus, two independent matching rooms were formed, which have visual and olfactory differences for rats. An infrared camera mounted on the top of the conditional position box, connected to an external computer, automatically recorded and analyzed the time (10 min) the rats spent in each compartment during the test. After each test, the conditional position box was cleaned with 10% ethanol.

(1) Preconditioning stage

In the Preconditioning stage (Day -1), after removing the clapboard, the rats were free to shuttle back and forth in the chamber, and the time (10 min) of the rats in compartment A and B was recorded as the baseline time in the pretreatment period. Rats with a clear natural preference (i.e., spending less than 30% of their time in a compartment) were removed.

(2) CFA conditioning stage

In the CFA Conditioning stage (Day 0), a clapboard was inserted to form two independent rooms. The rats were randomly placed in compartment "A" or compartment "B", and were allowed to move freely in

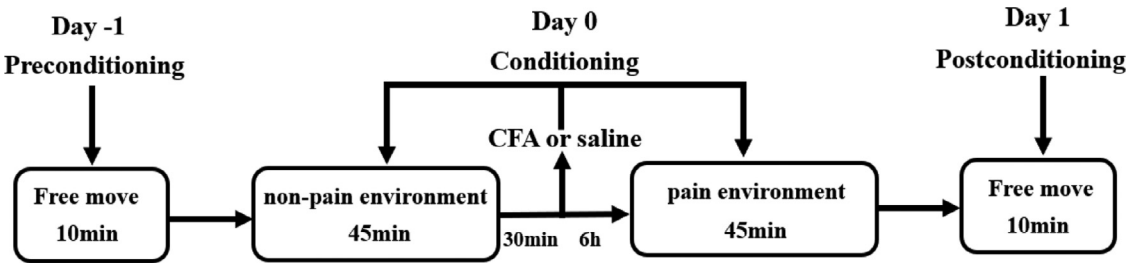

Fig. 2. C-CPA training process diagram.

these matching rooms for 45 min and placed back in cage. The matching room was defined as "non-conditional box" or "non-pain environment". After 30 min, the model group, the electroacupuncture group and the sham electroacupuncture group were injected with 0.1 ml Complete Freund's Adjuvant (Sigma, USA) into the left hind paw of rats, while the control group was injected with 0.9% sodium chloride at the same site. Six hours after injection, when the rats' hind paw showed signs of inflammation such as redness and swelling, the rats were put into another compartment to move freely for 45 min. The matching chamber was defined as "condition box" or "painful environment", and then the rats were put back into the cage.

(3) Postconditioning stage

In the postconditioning stage (Day 1), the clapboard was removed, and the rats were allowed to move freely for 10 min. The activity behaviors of the rats in two different compartments were recorded, and the residence time of the rats in the "non-pain environment" and "pain environment" was analyzed. If the rats' residence time in the "pain environment" was significantly reduced, it indicated that the c-CPA model was successfully established, and the rats showed pain aversion. Avoidance Score (CPA Score) = Postconditioning – Preconditioning. The training process of CPA is shown in Fig. 2.

2.4. EA treatment

All rats in the EA group were treated with EA stimulation. The intervention began at 30 min after CFA injection (Day 0), Stainless-steel needles (0.18 mm × 13 mm) were inserted into the bilateral Zusanli (ST36) and Kunlun (BL60) acupoints. The needles were connected to a HANS Acupuncture Point Nerve Stimulator (LH-202H Huawei Co, Ltd, Beijing, China). The parameters of the stimulator were as follows: 2/100 Hz, the intensity was set at 0.5 mA, 1.0 mA, and 1.5 mA (the intensity increased every 10 min, once a day).

Sham EA group: The same needles were inserted subcutaneously into the ST36 and BL60 acupoints (no more than 2 mm) of the animals. The needles were connected to the same stimulator, but no electrical stimulation was administered.

2.5. PWTs

The same up-down method was used in this experiment as reported previously (Xiang et al., 2019). The rats were placed into individual cages for 30 min before measurement to allow acclimation to the environment. The von Frey hairs (Stoelting Co, Thermo, Gilroy, CA, USA) were applied in a consecutive ascending order (0.4, 0.6, 1, 2, 4, 6, 8, 15, and 26 g) to the central surface of the hind paw (avoiding the footpad) until the hair bent into an "S" shape, and it was maintained there for 6-8 s. The force of 4 g was first applied. If the rat did not exhibit a positive avoidance response (such as brisk withdrawal or paw flinching), the result was recorded as "O", and the von Frey hair was replaced with a hair of higher force. Conversely, in the case of an avoidance response, the result was recorded as "X", and the von Frey hair was replaced with a hair of lower force. After a "OX" or "XO" combination of responses, 4 more measurements were performed and recorded as above (for example, "OXOXOO"). The pain threshold was calculated according to the following formula: PWTs (g) = $(10[Xf + \kappa\delta])/10000$, where "Xf" is the force of the last hair test, the "κ" value is obtained from the k-value table, and "δ" is the average value of the difference between the logarithm of hairs of each force, which is approximately equal to 0.231. Here, the maximum stimulation intensity was 26 g, and the minimum stimulation intensity was 0.4 g.

2.6. CPA test

The CPA test was conducted on Day 3. The whole test procedure and CPA Score calculation method was the same as that of postconditioning.

2.7. EZM

This EZM test was measured on a circular platform (100 cm diameter × 50 cm length × 25 cm width), consisting of two open arms and two closed arms. At the beginning of the test, rats were individually placed between the open arm and closed arm, facing the open arm with dim light and were allowed to explore the arena for 30 s. Rats activity was recorded on video during 5 min. The video was analyzed using Smart 3.0 software, including the data of the total distance, the distance in open arm, the time in open arm and the number of open arm entries. In order to eliminate the olfactory stimulus of the odor between animals, the test box was washed with 10% alcohol before each measurement to eliminate the information left by the previous rat such as odor, stool, etc.

2.8. Western blotting

Animals were anesthetized with 2% pentobarbital sodium and quickly perfused with 150 mL of 0.9% NaCl (4°C). Then, the ACC on the left side of the rat brain were extracted rapidly and stored at -80°C. The ACC was homogenized in RIPA buffer and centrifuged at 14000 rpm for 5 min at 4°C. The protein concentration of the tissue lysates was determined by the BCA method. 15 μg lysates were denatured and loaded and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany) by 5% SDS-PAGE electrophoresis. Next, the membranes were blocked with 5% low-fat milk in TBST for 1 h at room temperature. We used rabbit anti-GAD67 (1:200, Abcam) and rabbit anti-PV (1:200, Abcam) as the primary antibodies, and rabbit anti-GAPDH (HRP) (1:1000, Abcam) as the internal control. The membranes were incubated overnight at 4°C (> 12 h). Subsequently, the membranes were incubated with secondary antibody for 2 h at room temperature. An ECL kit (Pierce, Rockford, lL, USA) was used for development. The blots were photographed after color development, and the average optical density values of the bands were calculated.

2.9. Immunofluorescence

Rats were sacrificed under deep anesthesia with pentobarbital (80 mg/kg, i.p.) after the behavioral testing on Day 3. Rats were transcardially perfused with normal saline and 4% paraformaldehyde in 0.1 M

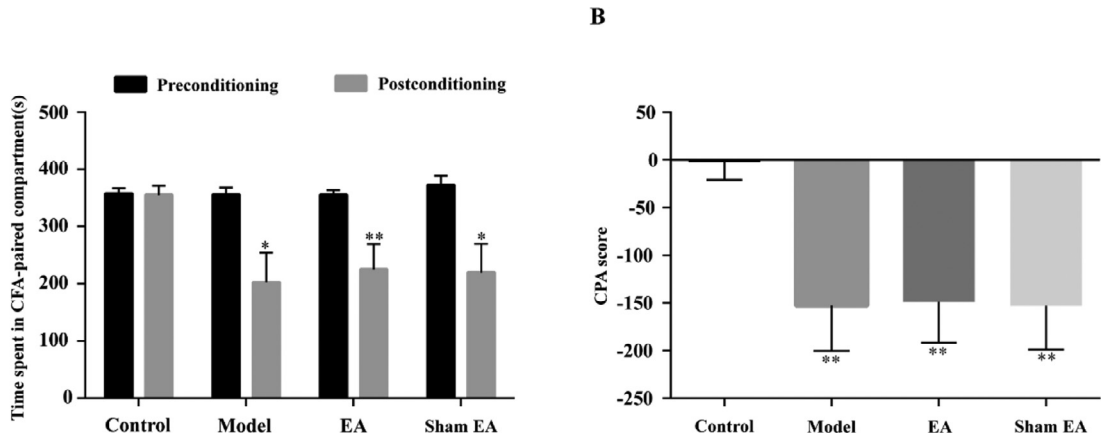

Fig. 3. The establishment of C-CPA model. (A) Time spent in the CFA-paired compartment on pre- and postconditioning days. (B) CPA scores on the postconditioning day. Data are presented as mean ± SEM, $^{*}P < 0.05$,$^{**}P < 0.01$ vs. Control.

phosphate-buffered saline (PBS, Solarbio, China) for prefixation. Then, the brain was postfixed in 4% paraformaldehyde for 24 h before transfer to 15% and 30% sucrose for dehydration. The OCT-embedded blocks were sectioned at 30 μm thickness. Sections were rinsed with TBST and blocked with 10% normal donkey serum for 1h at 37˚C. The sections were incubated overnight at 4˚C with the following antibodies: rabbit anti-NPY (1:400, ab10980, Abcam, USA). Subsequently, the sections were washed in TBST and incubated with the secondary Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488) preadsorbed (1:500, ab150061, Abcam, USA) for 1 h at 37˚C. Images were taken using the Imager. M2 microscope (ZEISS, Germany). The quantitative analyses of the number of positive cells in ACC were performed using the ImageJ software.

2.10. Statistical analysis

All data were presented as the mean ± standard error of the mean ($\bar{x}$ ± SEM). SPSS 20.0 software was used for statistical analysis. Analysis of variance (ANOVA) followed by LSD multiple comparison tests was used to compare three or more samples. Student's t-test was used to compare two independent samples. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The establishment of C-CPA model

As shown in Fig. 3A, compared with preconditioning, the time in the CFA-paired compartment on Day 1 was significantly reduced after the injection of CFA into the hind paw of the rats ($P < 0.05$), indicating the rats' aversion to conditioned place. In addition, as shown in Fig. 3B, all groups of CPA score showed significant decrease after CFA injection except the control group ($P < 0.01$). In conclusion, this result indicated the successful establishment of the C-CPA model, and pre-stimulation of EA for 2 days was not sufficient to relieve the pain aversion.

3.2. EA alleviates the mechanical hypersensitivity of C-CPA rats

The PWTs of rats were measured in each group on Day 0 and Day 2. As shown in Fig. 4, before the modeling, there was no significant difference in the PWTs between the groups ($P < 0.05$). After the injection of CFA (Day 2), there was a significant decrease in PWTs in the other groups compared with the control group. In addition, the PWTs of model group and sham EA group were significantly lower than that of the EA group ($P < 0.01$), indicating that the intervention of EA increased the pain thresholds in rats.

3.3. EA relieved pain aversion of C-CPA rats

To determine whether EA improved pain aversion in the C-CPA model, we performed CPA test. As shown in Fig. 5A, compared with preconditioning, rats in the model group and sham EA group of the time in the CFA-paired chamber had significantly reduced ($P < 0.05$), and the CPA score was significantly decreased compared with the control group ($P < 0.05$). However, compared with the model group, EA group could increase the time in the CFA-paired chamber and CPA score ($P < 0.05$), but sham EA group had no such effect. The results indicated that EA could relieve the pain aversion in C-CPA rats.

3.4. EA inhibited pain-induced anxiety behavior of C-CPA Rats

To further determine whether EA also interfered with pain-induced anxiety, we observed anxiety-like behavior changes with EZM test on Day 3. As shown in Fig. 6, compared with the control group, the distance in open arm, the time in open arm and the number of open arm entries were significantly reduced in the model group and the sham EA group ($P < 0.05$), while EA intervention could revert the consequence ($P < 0.05$). There was no difference in the total distance traveled for different groups of rats used in EZM test ($P > 0.05$), indicating that the motor ability of rats in each group was normal. The data showed that EA could relieve pain-induced anxiety behavior in C-CPA rats.

3.5. Effect of EA on the protein expression of GAD67, PV and NPY in ACC of C-CPA rats

To assess the intervention of EA on GABA-related proteins in ACC, we examined the protein expression of GAD67, PV and NPY in ACC of C-CPA rats. In Fig. 7, we found that only the protein level of NPY in ACC in the model group was significantly increased compared with the level in the control group ($P < 0.05$), while the levels of PV and GAD67 in the two groups showed no difference statistically ($P > 0.05$). Furthermore, the expression of NPY in ACC in EA group was significantly decreased compared with the model group and the sham EA group ($P < 0.05$), while there was no significant difference in PV and GAD67 ($P > 0.05$). It showed that C-CPA rats could increase the level of NPY, but not GAD67 and PV in ACC, and that EA could down-regulate the expression of NPY in ACC.

3.6. Effect of EA on the positive cells of NPY in ACC of C-CPA rats

To further examine the intervention of EA on NPY positive cells, we detected the number of NPY positive cells in ACC of C-CPA rats by immunofluorescence. In Fig. 8, we found that the number of NPY positive

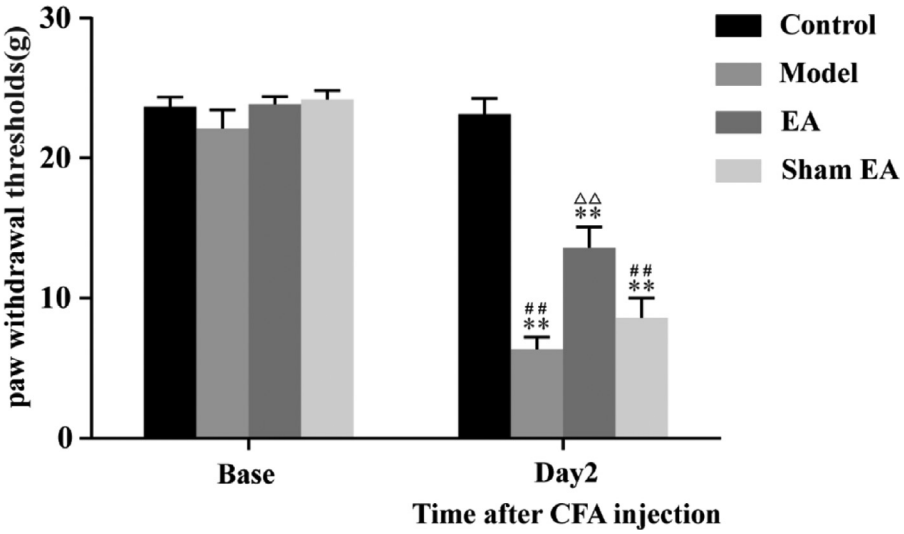

Fig. 4. Changes of paw withdrawal thresholds with EA treatment. Data are presented as mean ± SEM. $^{**}P < 0.01$ vs. Control; $^{\triangle\triangle}P < 0.01$ vs. Model; $^{\#\#}P < 0.01$ vs. EA.

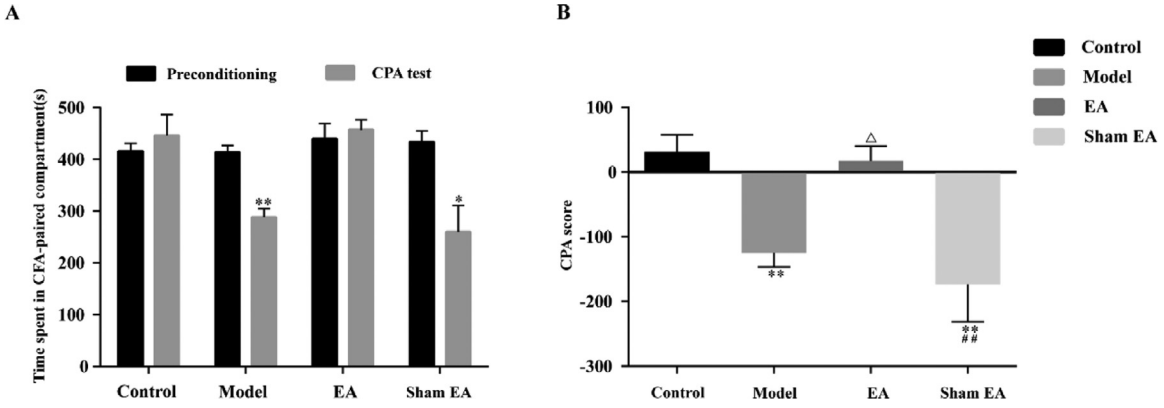

Fig. 5. Effects of EA stimulation on aversive emotion induced by the C-CPA model on Day 3. (A) Time spent in the CFA-paired compartment on the CPA test day in each group. (B) CPA scores on the CPA test. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control; $^{\triangle}P < 0.05$ vs. Model; $^{\#\#}P < 0.01$ vs. EA.

cells in ACC was significantly increased in the model group compared with those in the control group ($P < 0.05$). Compared with the model group, the number of NPY positive cells was significantly reduced in the EA group ($P < 0.05$). This was consistent with the results of Western blotting, suggesting that EA treatment can reverse the upregulated NPY in ACC of C-CPA rats. Sham EA had no effect on NPY expression in ACC.

4. Discussion

In our study we demonstrated that CFA-based pain aversion model is a reliable model for studying pain and pain associated anxiety-like behavior in rats. Using this model, we demonstrated that EA can effectively relieve the pain and pain-related emotions, and that mechanism of EA action may involve down-regulation of the expression of NPY in ACC.

Clinical studies have shown that avoidance behavior induced by pain emotions is a significant performance in the occurrence and development of chronic pain (Vlaeyen and Linton, 2012; Vlaeyen and Linton, 2000). Our research group used CFA-induced C-CPA model to study the relevant mechanism of pain emotion in rats. CFA-induced persistent hyperalgesia model is one of the most commonly used models of inflammatory pain (Ren and Dubner, 1999) and CPA is used to assess the state of aversive motivation during withdrawal of addictive drugs (Valero et al., 2018) and pain-induced aversion (Du et al., 2020). In our study we confirmed the establishment of affective pain based on the C-CPA model (Du et al., 2020). The study showed that after CFA was injected into the paw, and then after the “pain environment” matching conditionally, model group rats’ residence time in the “pain environment” matching box was significantly reduced in Day 1, indicating that the C-CPA model was successfully established. In addition, it is well known that the emotional changes caused by pain are diverse, and it is easy to cause anxiety, depression and other negative emotions. Few previous studies continued to observe pain anxiety on the basis of pain aversion. Therefore, in Day 3, we continued to use the EZM as a behavioral paradigm to detect the changes of pain anxiety in rats. The study found that the model group rats’ distance in open arm, the time in open arm and the number of open arm entries all decreased significantly, but there was no significant difference in total distance compared with the control group, indicating that the model rats had pain-induced anxiety, rather than surgery-induced motor impairment. The occurrence of pain-induced anxiety observed in the present study was significantly earlier than the result previously reported, when pain anxiety was commonly observed after 4 weeks (Yalcin et al., 2011). Because some studies have found that negative emotions can further aggravate the sensitivity of pain (Bushnell et al., 2013), and the C-CPA model established in this study can generate pain aversion. We speculated that the anxiety caused by pain may occur earlier because the generation of aversion increases the sensitivity to harmful stimuli.

Substantial evidence suggests that ACC plays an important role in processing harmful stimuli as well as the emotional dimensions associated with pain (Zhuo, 2016; Xiao and Zhang, 2018; Cao et al., 2014). Deep brain stimulation of the ACC in human study has been reported to relieve the emotional components of intractable chronic neuropathic pain (Boccard et al., 2017) and microinjection of excitatory amino acids

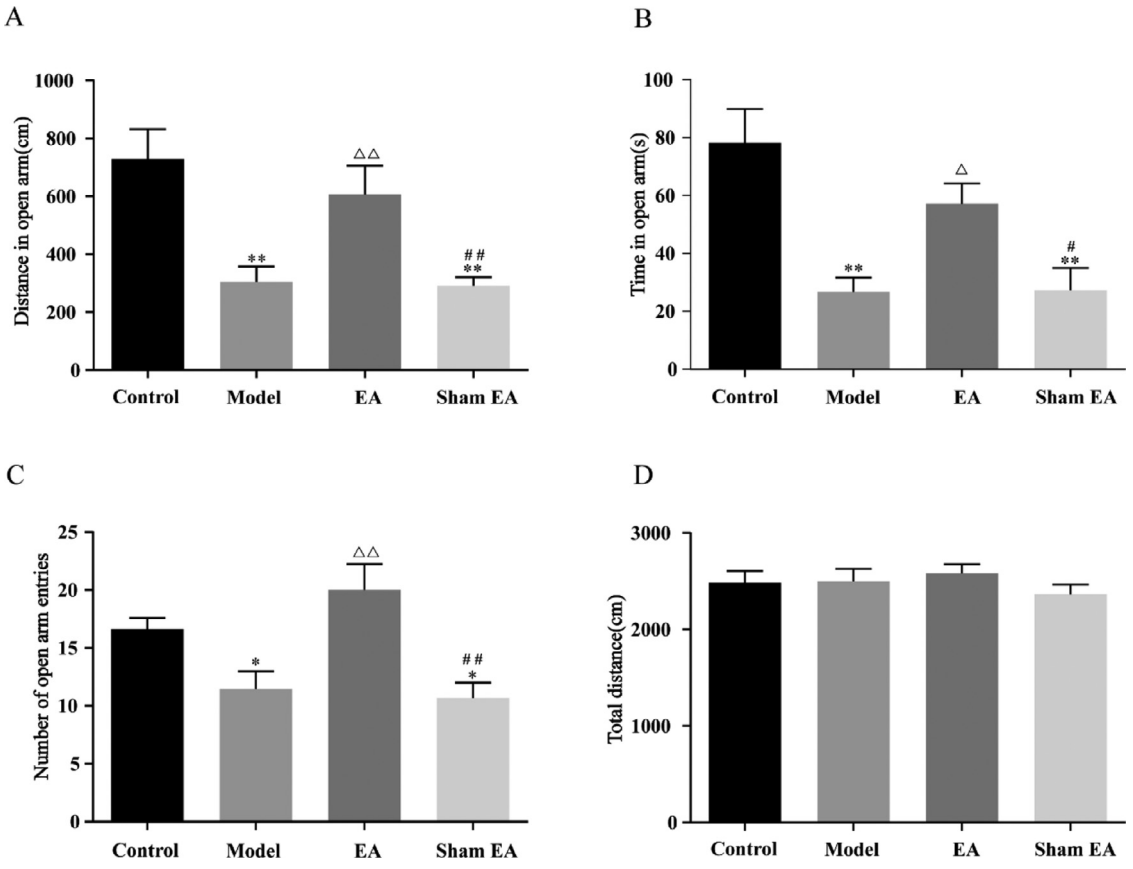

Fig. 6. Changes of EZM behavior with EA treatment. (A) the distance in open arm in each group. (B) the time in open arm. (C) the number of open arm entries in each group. (D) Total distance in the EZM in each group. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ vs. Model; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. EA.

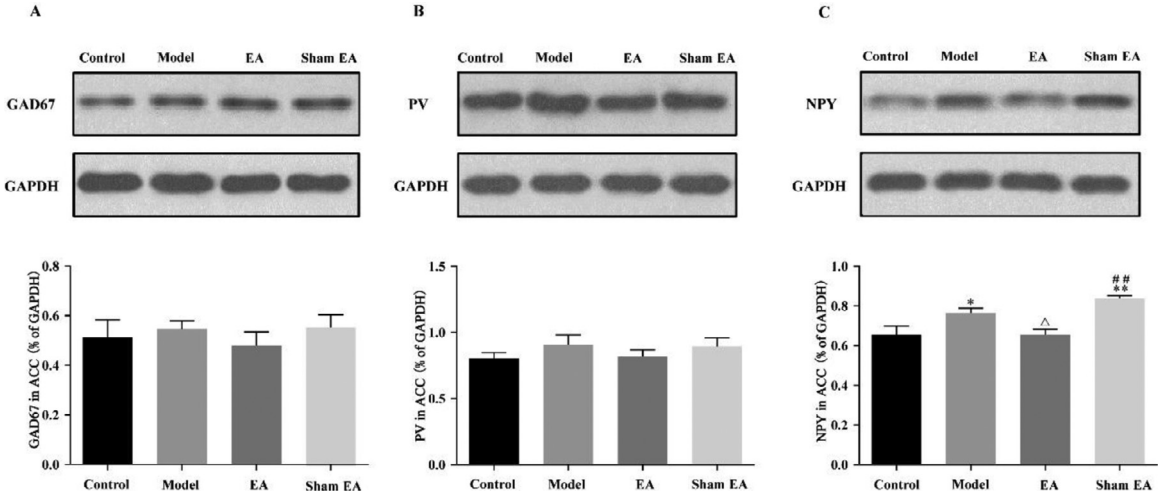

Fig. 7. The effect of EA treatment on the protein of GAD67, PV, and NPY in ACC of C-CAP rats. (A–C) Western blotting images and relative protein level of GAD67, PV, and NPY in rat from different groups. Dates are mean ± SEM; n =10. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control; $^{\triangle}P < 0.05$ vs. Model; $^{\#\#}P < 0.01$ vs. EA.

into ACC produced avoidance learning in the absence of a peripheral noxious stimulus (Johansen and Fields, 2004). In addition, a recent study in mice expressing rAAV-Camk2α-ChR2 showed that optogenetic stimulation of rostral ACC rather than mid cingulate cortex mediates pain affect or fear (Tan et al., 2017). While the exact neuronal correlates of transmission of pain associated affect remain to be established, a substantial amount of evidences support the role of GABAergic transmission in regulating anxiety (Lippa et al., 2005). Mechanism of action for some of the most prominent anxiolytic drugs involves potentiation of GABAA receptors (Mohler, 2012). Neuronal activity is however complex and depends on the balance of excitation and inhibition (E/I), through the complex interaction of glutamatergic and GABAergic interneurons (Page and Coutellier, 2019). A clinical study using magnetic resonance spectroscopy found inverse correlation between GABA

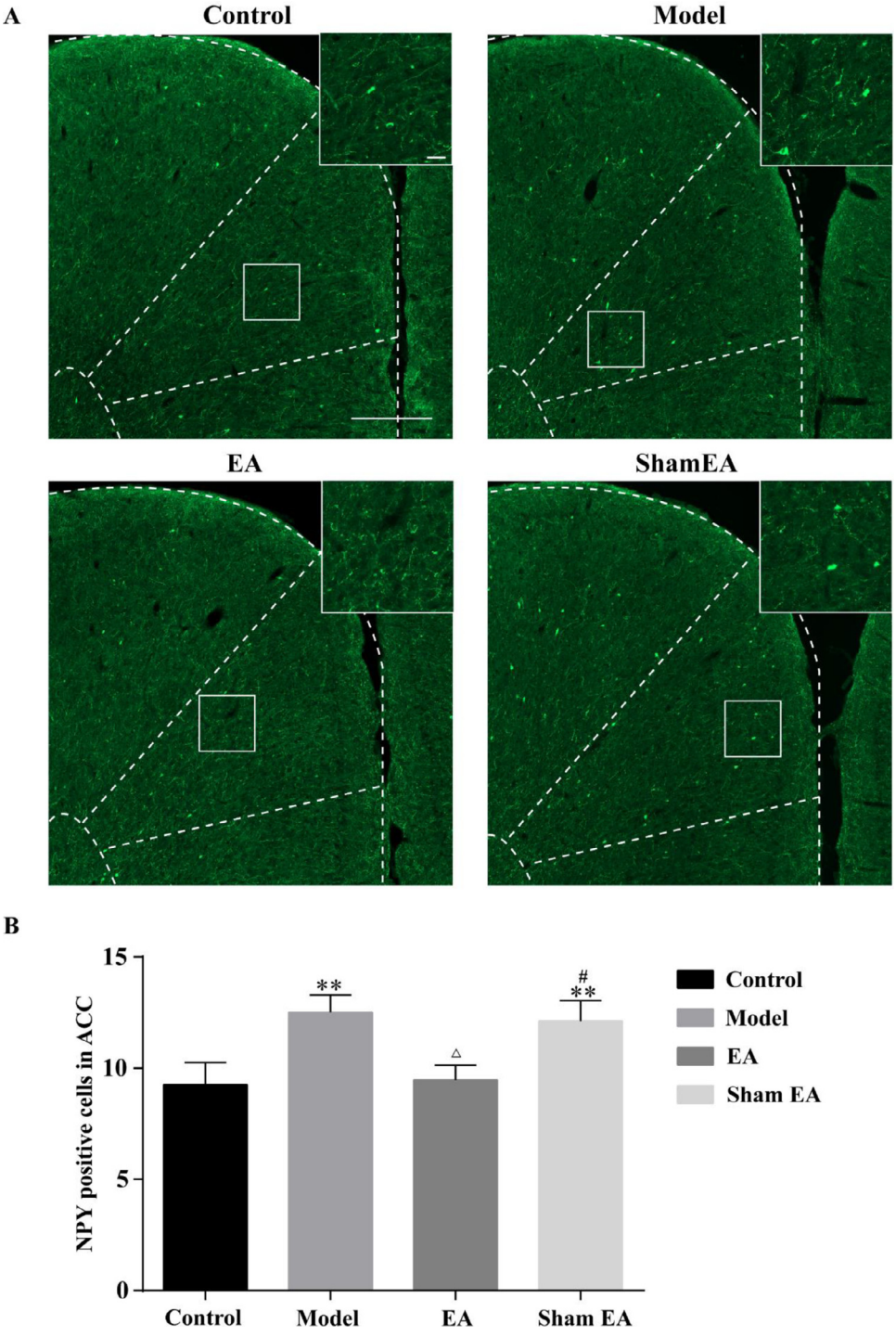

Fig. 8. The effect of EA treatment on the positive cells of NPY in ACC of C-CAP rats. (A) Representative images of NPY positive cells in the ACC in the different groups (scale bars: 500 μm; local figure scale bars: 50μm). (B) Quantification of the immunofluorescence results for NPY positive cells in the ACC in the different groups. Dates are mean ± SEM; $n = 3$–4. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control; $^{\triangle}P < 0.05$ vs. Model; $^{\#\#}P < 0.01$ vs. EA.

concentration and BOLD signal suggested that an increase in the activity of GABAergic interneurons led to a decrease in the activity of cortical pyramidal neurons and reduced excitatory drive to target nerve areas (Stan et al., 2014). Such notion is supported by electrophysiological recordings from rat ACC under condition of formalin-induced conditional positional avoidance (F-CPA) (Wang et al., 2005). The study revealed that the removal of GABAA inhibition with bicuculline enhanced glutamatergic transmission to ACC layer V neurons while activation of GABAA receptors with muscimol reduced glutamatergic transmission and blocked F-CPA without affecting formalin-induced acute nociceptive responses. Combined findings suggest that GABAA inhibition may be involved in pain-related aversive responses by modulating excitatory transmission in ACC. Another study reported that transplantation of inhibitory GABAergic interneurons progenitor cells into the ACC can block the position preference of gabapentin in neuropathic pain models (Juarez-Salinas et al., 2019).

Previous studies of our research group found multiple proteomic changes in ACC using CFA-induced CPA model. One study found that the GABA neurons of the ventral tegmental area inhibited dopamine neurons through the neurotransmission of GABAA receptors, thus causing conditional location avoidance (Tan et al., 2012). Other studies have reported removal of GABAA inhibition can promote glutamate reception-

mediated multisynaptic excitability transmission to ACC layer V neurons and activation of GABAA receptors in the ACC with muscimol blocked formalin-induced conditional positional avoidance, which suggested that GABAA inhibition may be involved in pain-related aversive responses by modulating excitatory transmission in ACC.

Glutamate decarboxylase 67 (GAD67), a key enzyme in GABA synthesis, can reflect the functional state of the GABA transmitter system in CNS. We have previously reported on multiple proteomic changes in ACC occurring in CFA-induced CPA model (Wu et al., 2019). In our current study, the Western blot analysis found no significant changes in GAD67 in ACC of C-CPA rats. Considering that pain-induced anxiodepressive-like consequences develop slowly and persist beyond the period of mechanical hypersensitivity (Sellmeijer et al., 2018), we suspect that early pain-induced emotions may not be sufficient to cause detectable changes in GAD67 expression. Our data, however, are in accordance with the observation in anxious mice under chronic stress, where the expression of GAD67 did not change in hippocampal tissue (Zhang et al., 2019).

Another neuropeptide with high expression level in the central nervous system and was associated with chronic pain and stress is NPY (Diaz-delCastillo et al., 2018; Reichmann and Holzer, 2016). An increase in NPY positive neurons in the medial prefrontal cortexof chronic stress-resilient rats was reported previously (Czeh et al., 2018), and a protective nature of such increase was suggested. NPY signaling in the parabrachial nucleus of the hindbrain can powerfully and selectively attenuate the behavioral response to inflammatory pain (Alhadeff et al., 2018). In addition, injection of NPY into the periaqueductal gray matter can produce analgesic and anti-anxiety effects (Vázquez-León et al., 2017). The changes in NPY levels of ACC and their relationship to emotion were to this date not explored in detail. Our study found that the expression protein and positive cells of NPY in ACC is significantly increased in the C-CPA model. The functional significance of increase in NPY levels and its effect on the regulation of pain and pain-associated emotion has, however, not been explored. It is noteworthy that the previous studies have found that low doses of celecoxib, diclofenac and duloxetine significantly inhibited the affective pain but not the sensory pain (Boyce-Rustay et al., 2010), suggesting that the emotional and sensory components of pain are supported by different mechanisms.

EA is a traditional Chinese medical treatment and has been proven to have beneficial effects on pain (Zhang et al., 2014; Liao et al., 2017) and pain-induced anxiety-like behaviors (Amorim et al., 2018; Liu et al., 2019) in both clinical studies and in preclinical research. Previous studies have also shown that acupuncture is a safe treatment with rare adverse side effects (White, 2004). In this study we first evaluated the effect of acupuncture in chronic inflammatory pain model in rats. In accordance with previously published data the EA intervention significantly reduced mechanical hypersensitivity (Xiang et al., 2019). To assess pain associated anxiety-like behavior in rats, we used a modified EZM device based on standard elevated cross maze (Tucker and McCabe, 2017) to assess the anxiety-like behaviors in the C-CPA model rats. Our main finding was that EA could alleviate the anxiety-like behaviors as assessed in the EZM (that is, increased the distance of open arm, the time in open arm and the number of open arm entries) and reduced the aversion (increased the spend time in the “painful environment”). In contrast, sham EA had no effect on either sensory or affective dimension. The therapeutic mechanism of EA is not fully understood, however, several previous studies explored the potential mechanism of EA in preventing anxiety-like and aversive behaviors. EA has been reported to reduce anxiety and depression produced by chronic unpredictable stress through reduced neuroinflamation in hippocampus (Yue et al., 2018). A different study reported that EA therapy alleviates adolescent cocaine exposure-enhanced anxiety-like behaviors in adult mice by attenuating the activities of PV interneurons in the prelimbic cortex (Nie et al., 2020). In our study, we found that EA reversed the increase of NPY protein level in ACC of C-CPA model rats, while it had no effect on levels of GAD67 and PV. Our finding is consistent with anxiolytic effects of NPY previously reported in animal models of anxiety (Vázquez-León et al., 2017).

5. Conclusion

In conclusion, our findings demonstrated that rat model of pain aversion could reliably induce anxiety-like behavior, and that EA effectively reverses chronic inflammatory pain and its negative emotions. The mechanism of EA action may involve downregulation of NPY expression in GABA ergic system of ACC. However, whether NPY was involved in both pain and pain emotion regulation remains to be further studied.

Ethical Approval

All animal care and experimental studies were approved by the Animal Care and Welfare Committee of Zhejiang Chinese Medical University, Zhejiang, China (Approval No. IACUC-20180319-12).

Data Availability

Date is available upon request to corresponding author.

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Declaration of Competing Interests

The authors declare no potential conflicts of interest.

CRediT authorship contribution statement

Fangbing Shao and Junying Du performed the data analysis and wrote the manuscript. Sisi Wang and Junfan Fang performed the experiments. Rok Cerne revised the manuscript. Xiaoming Jin and Jianqiao Fang designed the experiment. All authors contributed to the manuscript and approved the publication of the final manuscript.

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Supplementary Materials

Nil.

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