Proteomics analysis of the amygdala in rats with CFA-induced pain aversion with electro-acupuncture stimulation

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Background: Clinical patients suffering from pain usually exhibit aversion to pain-associated environments (pain aversion). Electro-acupuncture (EA) has been proven to be effective for the treatment of pain aversion in our previous studies. The amygdala could have substantial consequences on emotion and pain consolidation as well as general pain aversion behavior, however, the underlying mechanism remains unclear.

Purpose: The current study was performed to investigate Isobaric tags for relative and absolute quantitation (iTRAQ) based quantitative proteomic analysis of the amygdala in rats with complete Freund's adjuvant (CFA)-induced pain aversion, and comprehensive analysis of protein expression were performed to explore the underlying mechanism by which EA affects pain aversion.

Materials and methods: Inflammatory pain was induced with an intraplantar injection of 100 μL of CFA in the plantar surface of the left hind paw of the male Sprague-Dawley (SD) rats. Then the CFA-induced conditioned place aversion (C-CPA) test was performed. EA stimulation on the bilateral Zusanli and Sanyinjiao acu-points was used for 14 days and the EA stimulation frequency is 2 Hz. Based on iTRAQ-based proteomics analysis, we investigated the protein expression in the amygdala.

Results: EA can increase the paw withdrawal threshold in inflammatory pain induced by noxious stimulation. A total of 6319 proteins were quantified in amygdala. Of these identified proteins, 123 were identified in the pain aversion group relative to those in the saline group, and 125 significantly altered proteins were identified in the pain aversion + EA group relative to the pain aversion group. A total of 11 proteins were found to be differentially expressed in the amygdala of pain aversion and EA-treated rats. The expression of three proteins, glyceraldehyde-3-phosphate dehydrogenase, glutamate transporter-1, and p21-activated kinase 6, were confirmed to be consistent with the results of the proteome.

Conclusion: Our investigation demonstrated the possible mechanism of central nerve system by which EA intervention on pain aversion.

Keywords: pain, pain aversion, amygdala, electro-acupuncture, proteomics

Introduction

Pain is considered a multidimensional conscious experience that includes a sensory component (perception of the severity and location of the pain) and a negative affective-motivational component. Clinical patients suffering from pain usually exhibit varying degrees of affective symptoms such as aversion to pain-associated environments, which is defined as pain aversion. Recent surveys report that arthritis is one of the most common chronic pain conditions in North America, affecting approximately 16% of the adult population in the United States and Canada. The negative affective-motivational...
component of pain is different from the sensory component and amplifies the pain experience.\(^6\)\(^7\) Recently, a significant number of preclinical research groups have focused their attention on the affective symptoms of pain.\(^8\)\(^-\)\(^12\) However, the onset, development and maintenance of the affective component remain unclear.

The amygdala is part of the limbic system and plays important roles in emotion and pain formation, as well as depression and anxiety.\(^13\)\(^-\)\(^15\) Any malfunction and/or structural changes of the amygdala could have substantial consequences on emotion and pain consolidation as well as general pain aversion behavior.\(^16\)\(^,\)\(^17\) Amygdala gamma-aminobutyric acid (GABA)-A receptors appear to play an important role in sensory and especially affective pain processing in neuropathic rats.\(^18\) A state of pain may cause physiological changes in opioid transmission in the amygdala.\(^19\) Furthermore, TNF-\(\alpha\) contributes to the development of anxiety in mice with persistent inflammatory pain.\(^20\) Chronic forced swim stress has been shown to induce the activation of GluN2B-containing N-methyl-D-aspartate (NMDA) receptors and the sensitization of amygdala neurons.\(^21\) However, studies of emotional impairment induced by inflammatory pain are still relatively few in number, and the existing evidence is far from sufficient to clarify its inherent mechanisms.

Acupuncture, particularly electroacupuncture (EA), has been proven to be effective for the treatment of pain or emotional problems worldwide.\(^22\)\(^,\)\(^23\) Our previous studies have demonstrated that EA can increase the paw withdrawal threshold (PWT) in inflammatory pain and neuropathic pain induced by noxious stimulation.\(^24\)\(^,\)\(^25\) In recent years, the mechanism and treatment of psychiatric disorders associated with chronic pain have received increasing attention. Previous studies by our laboratory and others have reported that EA can significantly reduce the anxiety-like behaviors associated with chronic pain.\(^26\) However, the mechanism for these effects is unclear. We speculate that the effect of EA on pain aversion might be associated with the amygdala, although this has not been fully explored. Isobaric tags for relative and absolute quantitation (iTRAQ) is an isobaric labeling method used in quantitative proteomics by tandem mass spectrometry to determine the amount of proteins from different sources in a single experiment without provide other information. It uses stable isotope labeled molecules that can be covalent bonded to the N-terminus and side chain amines of proteins. Neuroproteomics via iTRAQ enables the holistic interrogation of functional changes at the molecular level following pain aversion and EA treatment.\(^27\)\(^,\)\(^28\)

Therefore, we hypothesize that the functional or structural components of the amygdala are involved in the formation of pain aversion and the effects of EA intervention. The current study was performed to investigate the effects of EA on the amygdala in rats with complete Freund’s adjuvant (CFA)-induced pain aversion. Specifically, comprehensive analysis of protein expression were performed to explore the underlying mechanism by which EA affects pain aversion.

**Materials and methods**

**Animals**

Male Sprague-Dawley rats (250±20 g) were purchased from the Shanghai Laboratory Animal Center (SLAC), Shanghai, China, and housed in 40×50×25 cm cages with ad libitum access to food and water at room temperature (25±1 °C). The animals were housed in groups of 5–6 rats with a 12:12-h light-dark cycle (dark cycle 8:00 PM-8:00 AM). Our experiments were approved by the Laboratory Animal Management and Use Committee of Zhejiang Chinese Medical University. All animal experiments were performed in accordance with the regulations of the State Science and Technology Commission for the Care and Use of Laboratory Animals (State Science and Technology Commission No. 2, 1988).

**Experimental design**

Rats were randomly divided into two groups: 1) a saline-injected group (saline, n=9) and 2) a CFA-injected group (CFA, n=18), with subgroups including the pain aversion and pain aversion + EA groups. On day 0, the baseline PWT was assessed before the saline/CFA injection and repeated on day 1, 7, 11 and 14. CPA tests were conducted on each rat on day 2 and were repeated on days 8 and 15. The timeline is shown in Figure 1.

**Arthritis pain model and sham controls**

Inflammatory pain was induced with an intraplantar injection of 100 \(\mu\)L of CFA in the plantar surface of the left hind paw of the rats. The injection contained 1 mg/mL heat-killed and dried Mycobacterium tuberculosis (ATCC 25177) in 0.85 mL of paraffin oil and 0.15 mL of mannide monooleate (Sigma, F5881, USA). Control animals were injected with the same volume of 0.9% saline.\(^8\)

**EA treatment**

Bilateral Zusanli (ST 36, 5 mm lateral to the anterior tubercule of the tibia) and Sanyinjiao (SP 6, 10 mm proximal to the prominence of the medial malleolus) acupoints were selected.
Stainless steel acupuncture needles (0.25 mm in diameter, 13 mm in length) were inserted into the acupoints at a depth of 5 mm. The two ipsilateral needles were connected to the output terminals of a Han’s Acupoint Nerve Stimulator (LH-202H, Huawei Co. Ltd., Beijing, China). The EA parameters were set as follows: square wave current output (pulse width: 0.2 ms); stimulation intensities of 1.0 mA, 1.5 mA and 2.0 mA, each one for 10 min in sequence, for a total of 30 min. The stimulation frequency was 2 Hz. All of the rats were loosely immobilized by a cloth cover with no physical restraint. EA was performed on day 2–15.

Conditioned place aversion paradigm
The CFA-induced conditioned place aversion (C-CPA) test was performed as previously reported using a place conditioning apparatus made of Plexiglas. The apparatus consisted of two equal square compartments, 30 cm on each side, positioned on the floor and separated by guillotine doors. No neutral third chamber was present in the center. One compartment was covered on three sides with 30 white dots, 1.8 cm in diameter, at 3.5-cm intervals; the floor of the compartment was covered with cinnamon oil. The other compartment was covered with 23 white equilateral triangles with a side length of 2.5 cm, and the floor of the compartment was covered with 5% acetic acid. The area of the compartment with 30 white dots was the same as the area of the compartment with 23 white triangles. The guillotine doors, covered with colored spots corresponding to their respective walls, were inserted during conditioning sessions and removed during pre- and post-conditioning tests. The colored dots and triangle spots served as visual cues, and the different substances on the floor served as olfactory cues. The testing room was illuminated with a 15-W bulb positioned approximately 1 m from the apparatus. The apparatus was cleaned with 75% ethanol after each test.

Preconditioning phase
In the preconditioning phase (day −1), the baseline time that the rats spent during a 15-min preconditioning period in each of the two distinctive compartments was recorded. The animal was considered to be in a chamber when the midpoint of the back was inside the chamber. The next day (day 0), each rat was free to explore one compartment for 1 h.

Conditioning phase
In the conditioning phase (day 1), each rat was free to explore one compartment for 1 h. For the C-CPA group, CFA was subcutaneously injected in the plantar surface of left hind paw (day 1). Two hours after the injection, the rat was free to explore another compartment for another hour. The pain-paired compartment was assigned randomly before the baseline measurement.

Testing phase
During the 15-min postconditioning phase (day 2), the rat was free to explore the both chamber for 15 min. The amount of time that the rats spent in each compartment was recorded again. The CPA score, used as an indicator of affective response, was determined by subtracting the time spent in the pain-paired compartment during the postconditioning test (day 2) from the time spent in the same compartment during the preconditioning test (day −1). Less postconditioning time spent in the compartment indicated a greater affective response. Additional tests were performed on days 2, 8 and 15. The timeline is shown in Figure 1.

Assessment of static mechanical sensitivity
The rats were habituated to the testing chambers two to three times before baseline testing began. The testing chambers consisted of clear Plexiglas chambers on a raised wire mesh grid. On each testing day, the rats were first habituated to the testing chambers 30 min before the test. Mechanical allodynia, as a behavioral sign of neuropathic pain, was assessed by
measuring the 50% paw withdrawal threshold (PWT) as described by Dixon.48

Mechanical threshold measurements for each hind paw were obtained. For this procedure, eight von Frey monofilaments (1.4, 2, 4, 6, 8, 10, 15 and 26 g) were utilized in the following manner. Each trial began with a von Frey force of 8 g delivered to the left hind paw for approximately 5 s. If there was no withdrawal response, the next higher force was delivered. If there was a response, the next lower force was delivered. This procedure was performed until no response was made at the highest force (26 g) or until four stimuli were administered following the initial response. The 50% PWT was calculated using the following formula: \( \text{PWT} = 10 \times [X_f + k] \), where \( X_f \) is the value of the final von Frey filament used (in log units), \( k \) is an obtained value from a given table according to the measured results from the pattern of positive/negative responses, and \( \delta = 0.184 \), which is the average interval (in log units) between the von Frey filaments. If an animal responded to the lowest von Frey filament, a value of 1.4 g was assigned. If an animal did not respond to the highest von Frey filament, the value was recorded as 15.0 g. The tests were conducted on days 0 (baseline), 1, 7, 11 and 14.

**Isobaric tags for relative and absolute quantitation (iTRAQ) proteomics analysis**

Extraction and digestion of proteins

Under deep anesthesia with 10% chloral hydrate (0.3 g/kg, i.p.), the rat brain was quickly extracted. The right amygdala was obtained according to the sixth edition of the Rat Brain in Stereotaxic Coordinates, and stored at −80 °C. SDT buffer (4% SDS, 100 mM Tris-HCl, 1 mM DTT, pH 8.0) was added to the sample. The lysate was homogenized, sonicated (4% SDS, 100 mM Tris-HCl, 1 mM DTT, pH 8.0) was added to the sample. The lysate was homogenized, sonicated and then boiled for 15 min. After the sample was centrifuged at 14,000 g for 40 min, the supernatant was collected every 1 min. The collected fractions were desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D.7 mm, volume 3 mL, Sigma) and concentrated by vacuum centrifugation.

The peptides were eluted at a flow rate of 1 mL/min with a gradient of 0–8% buffer B (500 mM KCl and 10 mM KH2PO4 in 25% of ACN, pH 3.0) for 22 min, followed by 8–52% buffer B during minute 22–47, 52–100% buffer B during minute 47–50, 100% buffer B during minute 50–58 min, and 0% buffer B after minute 58. The elution was monitored by absorbance at 214 nm, and fractions were collected every 1 min. The collected fractions were desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D.7 mm, volume 3 mL, Sigma) and concentrated by vacuum centrifugation.

**LC-MS/MS analysis**

Each fraction was injected for nano-liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 60 min. MS data were acquired using a data-dependent top-10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. The automatic gain control (AGC) target was set to 3e6, and maximum injection time to 10 ms. The dynamic exclusion duration was 40.0 s. Survey scans were acquired at a resolution of 70,000 at m/z 200, and the resolution for HCD spectra was set to 17,500 at m/z 200 with an isolation width of 2 m/z. Normalized collision energy was set to 30 eV, and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with peptide recognition mode enabled.

**Data analysis**

MS/MS spectra were searched using the MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4. The following parameters were set (Table 1).
Bioinformatics analysis
GO enrichment on three ontologies (biological process, molecular function, and cellular component) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were applied based on the Fisher exact test, considering the whole quantified protein annotations as the background dataset. Benjamini-Hochberg correction for multiple testing was further applied to adjust the derived p-values, and only functional categories and pathways with p-values under a threshold of 0.05 were considered significant. The relative protein expression data were used to perform hierarchical clustering analysis.

Western blot
Western blot was used to detect the protein expression levels of GAPDH, GLT-1, and PAK6 in the right amygdala (Figure S1). Protein samples (20 µg) from each group were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% nonfat milk followed by incubation with primary antibodies: rabbit anti-p-GAPDH (1:3000; ab181602; Abcam), rabbit anti-p-GLT-1 (1:500; ab106289; Abcam), rabbit anti-p-PAK6 (1:400; 13539-1-AP; Proteintech) and mouse anti-β-actin (1:5000; ab6276; Abcam). Then, the membranes were incubated with the secondary anti-rabbit IgG antibody H&L (1:20,000, ab6721; Abcam) or anti-mouse horseradish peroxidase (HRP)-conjugated IgG antibody H&L (1:20,000, ab205719; Abcam). The immunoreactive bands were visualized by using an Immun-Star™ HRP Chemiluminescence Kit (Bio-Rad). The intensity of each band relative to that for β-actin was measured by an ImageQuant LAS 4000 system (GE Healthcare, Hino, Japan) and was analyzed by ImageQuant TL software (version 7.0, GE Healthcare).

Statistical analysis
All data are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons among different groups were made by one-way repeated-measures analysis of variance (ANOVA). For the subsequent multiple comparisons, the least significant difference (LSD) and Dunnett’s test were used when equal variances were assumed or not assumed, respectively, as determined by the homogeneity of variance test.

Results
EA inhibits the mechanical hypersensitivity following CFA injection
Unilateral intraplantar injection of CFA solution into the left hind paw caused a decrease in the PWT after application of the mechanical stimulus to the ipsilateral side of the rat. Mechanical hypersensitivity was evident due to obvious paw swelling (Figure 2B) and significant decreases in the PWTs (Figure 2C) in the CFA-injected rats compared to those in the saline-treated rats (Figure 2A and C). In the saline group, the PWT exhibited a temporary decline and then recovered to the baseline level. Mechanical allodynia remained 14 days after the CFA injection, representing a significant decrease in the mechano-nociceptive threshold of the affected limb. Overall, CFA induced persistent mechanical allodynia in the CFA-treated hind paw.

Furthermore, we investigated the analgesic effects of EA on CFA-induced inflammatory pain by recording the PWTs of the left hind paws. EA significantly increased the PWT in CFA rats, with the CFA + EA rats showing a significant increase in PWTs from day 7 to day 14 after CFA injection (P<0.01).

EA inhibits the CFA-induced affective response in the C-CPA test
The CFA-induced conditioned place aversion (C-CPA) test was performed as previously reported using a place conditioning apparatus made of Plexiglas. During the 15-min preconditioning test, rats in the saline and pain aversion groups spent similar amounts of time in the two
compartments, indicating no preference for either compartment. However, when a hind-paw CFA injection was paired with a specific compartment, the rats spent less time in the paired compartment during the postconditioning test than during the preconditioning test, which demonstrated place aversion to the paired compartment. In contrast, saline-injected rats spent similar amounts of time in both compartments during the postconditioning and preconditioning tests, indicating no aversion to the saline-paired compartment (Figure 3A).

The CPA score revealed that the apparatus chambers were neutral stimuli and demonstrated that the CFA injection resulted in pain-induced place aversion compared to the saline injection. Meanwhile, EA significantly increased the time spent in the pain-paired compartment at day 15 ($P<0.01$, compared with the pain aversion group), which indicates that EA can relieve the pain aversion induced by CFA (Figure 3B).

**iTRAQ-based quantitative proteomic analysis of the amygdala in pain aversion rats**

To study the effect of EA on the protein expression in the amygdala of pain aversion rats, an enriched subfraction was prepared by a combination of fractionation and Triton solubilization. Next, we used differential isotopic labeling with the iTRAQ reagent to quantitate differences in protein levels. In the three groups, a total of 6319 proteins were identified and quantitated (with FDR <0.01). The change in the relative concentration of any given protein in the pain aversion and pain aversion + EA groups compared to that in the saline group was obtained from the iTRAQ 8-plex reporter ion ratios by a weighted average of all confidently identified peptides assigned to any given protein. iTRAQ reporter ratios of 1.2 and 0.83 were set as cut-off values for protein changes.

A total of 123 significantly altered proteins (either down- or upregulated) were identified in the pain aversion group relative to those in the saline group, all of which had at least one ratio with a $p$-value <0.05. Of these amygdala proteins, 24 were upregulated and 99 were downregulated (Table S1). These 123 differentially expressed proteins were annotated by Gene Ontology (GO) analysis, identifying 8 biological processes (Figure 4A): signal transducer activity, molecular function regulator, transcription factor activity, binding, molecular transducer activity, catalytic activity, structural molecule activity and transporter activity.

Meanwhile, a total of 125 significantly altered proteins were identified in the pain aversion + EA group relative to the pain aversion group (Table S2). Of these, 113 proteins were upregulated and 12 proteins were downregulated in the amygdala of EA-treated rats. As shown in Figure 4B, these 125 differentially expressed proteins were annotated by GO analysis, identifying 8 biological processes. The GO analysis chart shown in Figure 4C revealed that 11 proteins were altered in both the pain aversion and pain aversion + EA groups, indicating that these proteins may be the key proteins involved in the effects of EA intervention on pain aversion (Table 2).

**Variation in the differential proteins**

The identified differentially expressed proteins are involved in an extensive set of synaptic functions, including signaling,
Figure 3 The effect of EA on pain aversion in CFA rats. (A) Trajectory chart of rats in the preconditioning and postconditioning test treated with saline, CFA induction and EA stimulation. (B) The CPA was tested at baseline, day 2, day 8 and day 15. The CPA score was determined by subtracting the time spent in the pain-paired compartment during the postconditioning test. All data represent the mean ± SEM, n=9. **P<0.01, compared to the saline group; &&P<0.01, compared to the pain aversion group.

Table 2 11 proteins identified by iTRAQ altered in both pain aversion and pain aversion + EA groups

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regulatory, transporter, and trafficking functions. To further investigate the relationship between functional clusters and alterations in expression, each category of dysregulated proteins was systematically analyzed in the form of a heatmap (Figure 5). In displaying a summary of the altered proteins in each functional category, the heatmap emphasizes the unique dysregulation of protein expressions in the amygdala of the pain aversion and pain aversion + EA groups compared with the expression in the saline group. Eleven proteins were significantly downregulated in the pain aversion rats and upregulated in the pain aversion + EA group when compared to the expression in the saline rats, indicating that the two groups differed in their expression of several types of proteins.

We then validated the expression of the 11 proteins that were hypothesized to be functionally involved in pain aversion and EA treatment (Table 1) by Western blot (Figure S1). Finally, the changes in the expression of three proteins, D3ZGY4 (glyceraldehyde-3-phosphate dehydrogenase, GAPDH), Q8R462 (glutamate transporter-1, GLT-1), and D3ZQ51 (p21-activated kinase 6, PAK6), were confirmed to be consistent with the results of the proteome. The protein levels of GAPDH (Figure 6A), GLT-1 (Figure 6B) and PAK6 (Figure 6C) were downregulated in the amygdala of pain aversion rats but upregulated in the amygdala of the EA-treated pain aversion rats compared to the expression in the saline group (Figure 6).

Discussion
In the present study, plantar injection of CFA stimulated a rapid, local inflammatory response. The CFA-
induced pain was intense and persisted for at least 2 weeks after the injection. Moreover, the CFA injection induced more affective pain than the saline injection, as shown by the avoidance response to the pain-paired compartment over 2 weeks after the injection. EA, as a complementary and alternative medicine, exhibited an analgesic effect and inhibited the negative affective response caused by CFA-induced inflammatory pain as assessed by CPA.

We therefore investigated the effect of EA on protein expression in the amygdala. Based on iTRAQ-based proteomics analysis, a total of 123 significantly altered proteins were found in the pain aversion group, with 24 upregulated and 99 downregulated in the amygdala compared to the expression in the amygdala of the saline-treated rats. Meanwhile, a total of 125 significantly altered proteins were identified in the pain aversion + EA group, with 112 proteins upregulated and 13 downregulated in the amygdala of EA-treated pain aversion rats compared to the expression in the amygdala of the saline group. Finally, 11 proteins were altered in both the pain aversion and pain aversion + EA groups.

EA has widely been used in many countries to treat various diseases, including pain. Pain has multiple dimensions, including sensory discrimination and affective motivation. Many recent studies have demonstrated analgesic effects of EA on pain sensory discrimination and the underlying mechanisms. However, the effect of EA on pain aversion is less well studied. In an uncontrolled observation, acupuncture treatment had a more profound effect on the affective assessment than on the sensory assessment of pain. Previous studies have demonstrated that EA treatment significantly suppresses CFA-induced anxiety-like behaviors as assessed by the open-field test and elevated zero maze. Additionally, EA has been reported to attenuate the anxiety-like behavior induced by L5 spinal nerve ligation assessed by the elevated plus maze. These and our data suggest that EA can regulate the affective disorders caused by inflammatory pain. However, the mechanism for these effects remains unclear.

The amygdala, a key brain region linking pain sensation with negative emotions, presents a sensory and psychological dimension at the origin of affective and cognitive disorders. The amygdala has also emerged as an important brain center for the emotional-affective dimension of pain and for pain modulation. Hyperactivity in the laterocapsular division of the central nucleus of the amygdala accounts for pain-related emotional responses and anxiety-like behavior. Abnormally enhanced output from this area is the consequence of an imbalance between excitatory and inhibitory mechanisms. Recent studies have shown that a specific intrinsic neuromodulatory system within the amygdala, including glutamate, GABA, and noradrenaline neurons, is involved in the sensory and affective symptoms of persistent pain such as hypersensitivity to pain, anxiety- and depression-related behaviors, and fear extinction impairment. In this study, neuroproteomics revealed an interaction between EA on pain aversion and GAPDH, GLT-1, and PAK6 expression in amygdala. GAPDH is a ubiquitous enzyme that catalyzes the sixth step of glycolysis and, thus, breaks down glucose for energy.
Moreover, it is reported that the GLT-1 protein is expressed in the hippocampus, somatosensory cortex, striatum and amygdala, which exercises important influences on bipolar disorder, depression, Alzheimer disease and epilepsy. For instance, Xiaoling Zhu found that down-regulation of astroglial glutamate transporter-1 impairs pain-evoked endogenous analgesia in rats. In our research, the decreased GLT-1 of pain aversion rat was upregulated by EA, however, the exact mechanism remains unclear.

In our study, PAK6 in the amygdala was also found to be involved in the intervention of EA on pain aversion. Finally, PAK6 is a member of the group B family of PAK serine/threonine kinases and is highly expressed in the brain. PAK6 is a downstream effector of Rho GTPases and can affect a variety of processes in different cell types and tissues by remodeling the cytoskeleton and by promoting gene transcription and cell survival. But no relevant literature about PAK6 was reported in the field of pain, depression or anxiety.

In summary, our data demonstrated that inflammatory pain exposure resulted in pain-induced place aversion, which leads to comprehensive biological changes in the amygdala. Based on proteomics analysis, a total of 11 proteins were found to be differentially expressed in the amygdala of pain aversion and EA-treated rats. GO and Western blot analyses verified that GAPDH, GLT-1 and PAK6 expression were related to the effects of EA on pain aversion. One of the possible mechanisms by which emotion modulation is impaired by inflammatory pain may be related to glutamate excitotoxicity and imbalances in specific neurotransmitters. Thus, our study paves the way for further examining the functional role of EA influence the activity of amygdala in pain aversion.

![Figure 6 GAPDH, GLT-1 and PAK6 expressions in rat amygdala induced by EA. GAPDH, GLT-1 and PAK6 level in amygdala of the Saline, Pain aversion and Pain aversion + EA rats was quantified by Western blot normalized to β-actin level (GAPDH 36KD, GLT-1 62KD, PAK6 60KD, β-actin 42KD). Quantitative analysis of Western blot (n=3, *p<0.05, compared to the saline group; **p<0.01, compared to the saline group; ***p<0.01, compared to the pain aversion group. Full-length blots/gels are presented in Figure S1. The samples derive from the same experiment and that gels were processed in parallel. The grouping of gels/blots cropped from different parts of the same gel.](https://www.dovepress.com/doi-image)
Data availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work. Jianqiao Fang and Yuanyuan Wu conceived and designed the experiments. Xiaofen He, Zui Shen and Yan Shi performed the behavioral tests. Yongliang Jiang and Xiaomei Shao analyzed the results and wrote the paper. All authors read and approved the manuscript.

Disclosure
The authors report no conflicts of interest in this work.

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