The volatile oil from Acori Graminei Rhizoma downregulates NMDARs-ERK1/2-CREB signaling pathway to alleviate pain related emotions induced by inflammatory pain in rats

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Abstract  Objective  To investigate whether Acori Graminei Rhizoma (AGR) volatile oil can reduce inflammatory pain-induced pain-related mood in rats by downregulating NMDARs-ERK 1/2-CREB signaling pathway.  Methods  Thirty-six male SD rats were randomly divided into six groups: control group, sham group, complete Freund's adjuvant (CFA) group, CFA + 0.1 mL/10 g volatile oil group, CFA + 0.2 mL/10 g volatile oil group, and CFA + 0.4 mL/10 g volatile oil group. Six rats in each group were gavaged for 21 days and tested for mechanical pain hypersensitivity and positional avoidance (PEAP) before and after drug treatment. In addition, another batch of thirty-six male SD rats were randomly divided into six groups, including control, sham, CFA, CFA + saline, CFA + 0.1 mg/kg NMDA, and CFA + 0.1 mg/kg Ifenprodil groups. The control group did not receive any treatment, and the sham group received the same volume of sterile saline at the same site. Animals in each group were tested for mechanical pain hypersensitivity and positional avoidance before and after drug treatment. The expression levels of NMDAR1, NMDAR2A, NMDAR2A2B, mGlur1, ERK 1/2, CREB, and c-Fos in the basolateral amygdala (BLA) were determined by immunofluorescent staining and Western blot methods.  Results  AGR essential oil could reduce mechanical hyperalgesia and position avoidance in animals caused by CFA, and down-regulate NMDARs, mGlur1/5, ERK 1/2 and CREB expression. While up-regulation of c-Fos expression by NMDAR inhibitor Ifenprodil, reversed the above phenomena.  Conclusion  AGR essential oil alleviates inflammatory pain-induced pain-related mood in rats through downregulation of the NMDARs-ERK1/2-CREB signaling pathway.

Key words  Inflammatory pain; Pain-related emotions; NMDAR; ERK; CREB

1 Introduction

Chronic pain is a major public health issue with an incidence of 20%-25%, which brings a huge economic burden to countries all over the world[1-2]. Inflammatory pain is a chronic pathological pain induced by inflammation caused by trauma, bacterial or viral infections. The main clinical manifestations include hyperalgesia, touch-
induced pain and spontaneous pain, accompanied by symptoms such as anxiety, depression, insomnia and quality of life decline\cite{3-4}. Clinical patients suffering from pain usually show different degrees of emotional symptoms, such as aversion to pain-related environment\cite{5-6}, which causes great harm for the patients. At present, NSAIDs are among the most popular over-the-counter drugs across the world, constituting 5% of all the prescribed medicines, but long-term use of this drug may cause serious adverse reactions\cite{7-8}. What's more, there are relatively few reports on affective-motivational pain responses caused by inflammatory pain. Therefore, it is extremely urgent to find novel and effective methods to treat affective-motivational pain responses caused by inflammatory pain.

Glutamate is one of the dominant excitatory neurotransmitters and one of its receptors, \(N\)-methyl-\(D\)-aspartate receptor (NMDAR), is a member of the ionic glutamate receptor family, which not only facilitates fast excitatory synaptic transmission in the central nervous system\cite{9} but also is a key molecule for regulating pain perception\cite{10}. NMDARs exist in the presynaptic nerve terminals which can affect synaptic transmission and neuronal plasticity in the amygdala, hypothalamus, striatum and hippocampus, and participate in mediating the transmission and integration of noxious information\cite{11}. NMDARs are involved in the central sensitization of pain and mediate hyperalgesia and hyperalgesia produce\cite{12}, which play an important role in the plasticity of the amygdala-dependent fear regulation basis. Studies find that glutamatergic projection from the insular cortex (IC) to the basolateral amygdala (BLA) is critical for the formation of observational pain\cite{13}. Therefore, whether NMDARs are involved in the generation of inflammatory pain in the basolateral amygdala (BLA), or whether they are involved in the formation of affective-motivational pain responses require further research.

\textit{Acori Graminei Rhizoma} (AGR) as a traditional herbal medicine, the dry rhizoma of Acorus gramineus Soland, has been used in the therapy of cerebrovascular diseases, Alzheimer's disease\cite{14} and various inflammatory diseases\cite{15}. While volatile oil is the main active ingredient of AGR, including \(\alpha\)-asarone, \(\beta\)-asarone, etc. These components can quickly enter brain tissue through the blood-brain barrier\cite{16}, and are widely distributed in the brainstem, hippocampus, cortex and cerebellum\cite{17}. Studies have reported that AGR extract could reduce thermal hyperalgesia, thermal allodynia and mechanical hyperalgesia of neuropathic pain model rats\cite{18}. \(\beta\)-asarone could significantly improve spatial memory and synaptic plasticity caused by chronic inflammation and downregulated the expression of inflammatory cytokines\cite{19}. Previous research by the research team found that AGR can reduce the activation of astrocytes in BLA of rats with chronic inflammatory pain induced by CFA (complete Freund's adjuvant, CFA), and further inhibit the expression of \(c\)-Fos, but the specific mechanism was not explored. And whether volatile oil can effectively relieve affective-motivational pain responses caused by inflammatory pain and related mechanisms are still unclear. Therefore, this study adopted a complete Freund's adjuvant (CFA)-induced inflammatory pain model and took CFA inflammatory rats as the research object to further explore the mechanism of volatile oil from AGR, in order to provide experimental evidence for the relief of affective-motivational pain responses induced by inflammatory pain.

2 Materials and methods

2.1 Animals

Seventy-two male Sprague-Dawley rats weighing 200-230 g were respectively obtained from Guangdong Medical Lab Animal Center, China (Permit Number: SCXK GUANGDONG 2018-0034). The animals were housed in separate cages with controlled humidity (55% ± 5%) and
temperature (25 ± 1) °C under an even dark/light cycle. Food and water were available ad libitum throughout the whole experiment. Before the start of the experiment, rats were allowed to adapt to the new environment for a week. All procedures were approved by the Animal Ethics Committee of Guangdong Pharmaceutical University [SCXK (Guangdong) 2018-0034] and were consistent with the National Institute of Health Guide for the Care and Use of Animals in Research. All experimental studies were conducted to minimize the use and discomfort of animals.

2.2 Construction of rat model of inflammatory pain

After anesthesia with 2% isoflurane, the animals positioned the right lateral malleolus of the fibula and quickly and vertically penetrated the skin with a 22-gauge needle. The needle was rotated and inserted into the joint cavity between the tibiofibular and tarsus bone until a loss of resistance was felt\(^{[20]}\). After being injected with CFA (50 μL), the needle was withdrawn and then the pinhole was pressed for a while.

2.3 Intra-BLA injection

On the 10th day after the operation, the animals were anesthetized with 4% chloral hydrate (100 g/mL b.w., i.p.) and stereotaxic surgery was performed according to the rat brain atlas. The scalp was shaved and a 1.0 cm midline sagittal incision was made to expose the skull and bregma. Two tiny holes were drilled into the skull, NMDA (4 μL) and Ifenprodil (4 μL) were respectively injected into the BLA region of amygdala at the following coordinates from bregma, AP: −2.8 mm, ML: ±5.0 mm, DV: −8.6 mm according to the rat's brain atlas of Paxinos and Watson using a 5 μL microsyringe (Hamilton) driven by a minipump at a rate of 0.2 μL/min. Keep the needle for another 5 min after injection, and then sutured the incision. The sham rats underwent the same procedure except that sterile saline was injected with the same volume as the NMDA and Ifenprodil solution. The control rats did not undergo the surgical procedure, nor received any injections.

2.4 Drugs, animal treatment and experimental design

CFA (F5881) was bought from Sigma company. The volatile oil of AGR was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. The volatile oil was diluted by 1% tween-80 to 100 times before oral gavage for rats\(^{[21]}\). NMDAR agonist (NMDA) and NMDAR antagonist (Ifenprodil) were bought from ApexBio Technology Co., Ltd.

Experiment 1: Thirty-six male SD rats were randomly divided into six groups, the control group, sham group, CFA group, CFA + 0.1 mL/10 g b.w. volatile oil group, CFA + 0.2 mL/10 g b.w. volatile oil group and CFA + 0.4 mL/10 g b.w. volatile oil group. Six rats in each group were taken gavage for 21 consecutive days, and these animals were tested for behaviors related to affective-motivational pain responses before and after drug treatment (Fig. 1A).

Experiment 2: Thirty-six male SD rats were randomly subdivided into six groups with six animals per group, the control group, sham group, CFA group, CFA + saline group, CFA + 0.1 mg/kg NMDA group and CFA + 0.1 mg/kg Ifenprodil group. The control group received no treatment and the same volume of sterile saline was injected into the same site in the sham group. The animals in all groups were tested for behaviors related to affective-motivational pain responses before and after drug treatment (Fig. 1B).

2.5 Mechanical allodynia test

A set of von Frey filaments were used to measure mechanical allodynia in rat hind paws by measuring the paw withdrawal threshold (PWT) using the "up and down" method as previously
described\textsuperscript{[22-24]}. First, rats were placed in separate plexiglass chambers positioned on a mesh table to adapt the test environment. The withdrawal threshold (in grams) to mechanical stimulation was examined for both the ipsilateral and contralateral hind paws before baseline and at 1, 3, 5, 7 days after CFA or saline injection. A single von Frey fiber was applied to the plantar surface for five times with an inter-stimulation interval of 5 s. The positive response was defined as at least one clear paw withdrawal response out of the five applications.

2.6 Place escape/avoidance paradigm (PEAP) test

PEAP test was assessed as a behavioral indicator of avoidance of pain aversion using light area time/experimental time (%). This experiment allowed the inflammatory pain model to induce pain-related emotional behaviors in rats. The test was conducted as previously described by Labuda and Fuchs\textsuperscript{[25]}. Rats were placed within the plexiglass chamber ($25\times30\times30$ cm) positioned on the top of a mesh screen. One half of the chamber was transparent (light area) and the other half of the
chamber was black (dark area). The lateral plantar surface of the rat was stimulated with von Frey fiber (60 g) from the box bottom (15 s/stimulation, 20 min) after the initial stimulation. Then the affected plantar was stimulated in the dark area, whereas the healthy plantar surface was stimulated in the bright area. The time of rats in the bright area was recorded. Bright area time/experiment time (%) was used as a behavioral index to escape pain aversion.

2.7 Immunofluorescence staining

Rats were deeply anesthetized with 4% chloral hydrate (100 g/mL b.w., i.p.) and transcardially infused with 0.9% saline and 4% polyformaldehyde in 0.01 M phosphate buffer solution (PBS, pH 7.4, 4 ℃). The brains were sampled, fixed in the same fixative solution overnight 48 h at 4 ℃, and washed with running water, dehydrated with gradient alcohol, treated with xylene and wax. The brain was cut into coronal sections (4-6 μm thick). After being dewaxed and dehydrated, brain sections were immersed in 0.01 M citrate buffer (pH 6.0) and heated for 15 min to activate antigens. The goat serum was added for 45 min and then incubated with the following primary antibodies (0.3% Triton X-100, Abcam), rabbit anti-NMDAR1 (1:100, Abcam), rabbit anti-NMDAR2A (1:100, Abcam), rabbit anti-NMDAR2B (1:100, Abcam), rabbit anti-metabotropic glutamate receptors (mGlur1) (1:100, Abcam), rabbit anti-mGlur5 (1:100, Abcam), rabbit anti-ERK1/2 (1:100, Cell signaling), rabbit anti-CREB (1:100, Cell signaling), rabbit anti-c-Fos (1:100, Abcam). Sections were rinsed with PBS for 3 times (5 min/time) and then incubated with the following primary antibodies (0.3% Triton X-100) overnight at 4 ℃ respectively: rabbit anti-NMDAR1 (1:100, Abcam), rabbit anti-NMDAR2A (1:100, Abcam), rabbit anti-NMDAR2B (1:100, Abcam), rabbit anti-metabotropic glutamate receptors (mGlur1) (1:100, Abcam), rabbit anti-mGlur5 (1:100, Abcam), rabbit anti-ERK1/2 (1:100, Cell signaling), rabbit anti-CREB (1:100, Cell signaling), rabbit anti-c-Fos (1:100, Abcam). Membranes were blocked with 5% skim milk for 1.5 h at room temperature and incubated overnight with primary anti-bodies targeting the following proteins at 4 ℃: rabbit anti-NMDAR1 (1:1 000, Abcam), rabbit anti-NMDAR2A (1:1 000, Abcam), rabbit anti-NMDAR2B (1:3 000, Abcam), rabbit anti-mGlur1 (1:1 000, Abcam), rabbit anti-mGlur5 (1:5 000, Abcam), rabbit anti-ERK1/2 (1:1 000, Cell signaling), rabbit anti-CREB (1:1 000, Cell signaling), rabbit anti-c-Fos (1:1 200, Abclonal). Membranes were incubated with HRP-conjugated secondary antibody for 2 h at room temperature: horse-radish peroxidase AffiniPure goat anti-rabbit IgG (1:10 000, Earthox). The membranes were washed with TBST 3 times. Blots were visualized by automatic chemiluminescence imaging and analysis system (Tanon, Shanghai, China) and calculated the ratio of optical density of each protein to GAPDH (1:10 000, Abcam). All the experiments were repeated at least triplicate.

2.8 Western blot analysis

Rats were decapitated after being anesthetized with 4% chloral hydrate, the BLA was extracted from the brain immediately and then stored at −80 °C before use. The tissues were homogenized in a pretreated lysis buffer with protease inhibitors. The homogenate was centrifuged at 12 000 rpm at 4 ℃ for 15 min. The supernatant was reserved for subsequent experiments, and the protein concentration was determined using the bicinchoninic acid Protein Assay Kit (Beyotime, Shanghai, China). Then protein samples were separated on SDS-PAGE gels (6%, 8%, 10% gradient gel) and transferred to PVDF membranes. Membranes were blocked with 5% skim milk for 1.5 h at room temperature and incubated overnight with primary anti-bodies targeting the following proteins at 4 ℃: rabbit anti-NMDAR1 (1:1 000, Abcam), rabbit anti-NMDAR2A (1:1 000, Abcam), rabbit anti-NMDAR2B (1:3 000, Abcam), rabbit anti-mGlur1 (1:1 000, Abcam), rabbit anti-mGlur5 (1:5 000, Abcam), rabbit anti-CREB (1:1 000, Abcam), rabbit anti-CREB (1:1 000, Cell signaling), rabbit anti-c-Fos (1:1 200, Abclonal). Membranes were incubated with HRP-conjugated secondary antibody for 2 h at room temperature: horse-radish peroxidase AffiniPure goat anti-rabbit IgG (1:10 000, Earthox). The membranes were washed with TBST 3 times. Blots were visualized by automatic chemiluminescence imaging and analysis system (Tanon, Shanghai, China) and calculated the ratio of optical density of each protein to GAPDH (1:10 000, Abcam). All the experiments were repeated at least triplicate.
3 Results

3.1 Volatile oil of AGR alleviates CFA-induced mechanical hyperalgesia and place-escape/avoidance

Pain and emotional behavior tests were performed on days 1, 3, 5 and 7 after injection of CFA. There was a significant difference between the CFA group and control and sham group after the operation and on days 1, 3, 5 and 7 (Fig. 2A, 2B; P<0.05). Compared with the control group, rats in the CFA group had a lower mechanical nociceptive threshold on the contralateral hind paw on days 1, 3, 5 and 7 after surgery (Fig. 2A; P<0.05). The mechanical nociceptive thresholds of the rats in the CFA group on the ipsilateral hind paw were significantly reduced on days 1, 3, 5 and 7 after the operation (Fig. 2B; P<0.01). And the CFA group stayed in the light area for a significantly longer time than the control and sham group (Fig. 2E; P<0.01). These data indicated that CFA-induced allodynia and mechanical stimulation of painful hind paw had an avoidance effect on the preferred location.

To determine whether volatile oil of AGR could relieve CFA-induced mechanical hyperalgesia and place escape/avoidance, the behavioral tests were performed again on days 1, 3, 5, and 7 after volatile oil treatment. Compared with the control group, the CFA rats had a significant reduction in the contralateral and ipsilateral mechanical nociceptive thresholds on days 1, 3, 5 and 7 after volatile oil treatment (Fig. 2C-D; P<0.01). The CFA rats stayed at the percent of time spent in the light chamber was significantly increased (Fig. 2F; P<0.01). After being treated with volatile oil, the percent of time spent in the light chamber of animals was reduced on day 1, 3, 5 and 7, especially in the CFA + 0.2 mL/10 g b.w. volatile oil group and the CFA + 0.4 mL/10 g b.w. volatile oil group (Fig. 2F; P<0.01). These results indicated that administration of CFA could induce mechanical hyperalgesia and avoid the preferred position of mechanical stimulation of the painful hind paw, while treatment of volatile oil highly attenuated the CFA-induced mechanical hyperalgesia and the avoidance of the preferred position of mechanical stimulation of pain.

3.2 Volatile oil of AGR alleviated the toxic effects of NMDARs and mGlur1/5

Studies have shown that there is a synergistic effect between NMDAR and mGlur1/5\(^{[26]}\). Studies have also shown that mGlur1 and mGlur5 can increased the activity of NMDA receptors\(^{[27]}\). The immunofluorescent results showed that positive expressions of NMDAR1, NMDAR2A, NMDAR2B, mGlur1 and mGlur5 in the BLA of the control and sham rats were decreased, and there were no significant differences between the two groups (Fig. 3C-D; Fig. 4D-G; Fig. 5D-G; all P<0.05). Compared with the control and the sham group, the CFA rats showed obvious expressions of NMDAR1, NMDAR2A, NMDAR2B, mGlur1 and mGlur5 in the BLA (Fig. 3C-D; Fig. 4D-G; Fig. 5D-G; all P<0.05). There was no significant difference in the expressions of NMDAR1, NMDAR2A, NMDAR2B, mGlur1 and mGlur5 between CFA group and the CFA + 0.1 mL/10 g b.w. volatile oil group. The expressions of NMDAR1, NMDAR2A, NMDAR2B, mGlur1 and mGlur5 in the CFA + 0.2 mL/10 g b.w. volatile oil group and the CFA + 0.4 mL/10 g b.w. volatile oil group were significantly reduced when compared to CFA group (Fig. 3C-D; Fig. 4D-G; Fig. 5D-G; all P<0.05). The results of western blot were further confirmed the above immunofluorescent results. Compared with
Fig. 2  Volatile oil of Acori Graminei Rhizoma alleviates CFA-induced mechanical hyperalgesia and position avoidance.

Pain and emotional behavior were tested on the first 1, 3, 5, and 7 days after CFA injection. A. Contralateral mechanical pain withdrawal thresholds of rats; B. Ipsilateral mechanical pain withdrawal thresholds of rats; C. Contralateral mechanical pain withdrawal thresholds; D. Ipsilateral mechanical pain withdrawal thresholds; E. Percent of time within light side of chamber in Post-operative days; F. Percent of time within light side of the chamber in Post-treatment days. n=6. *P<0.05, **P<0.01 in figure A, B: CFA group vs control and sham group. ***P<0.01 in figure C, D, F: CFA group vs control group, CFA group vs CFA + 0.2 mL/10 g b.w. volatile oil group. "**P<0.01 in figure C, D, F: CFA group vs CFA + 0.4 mL/10 g b.w. volatile oil group.
the control group, the expression of NMDAR1, NMDAR2A, NMDAR2B, mGluR1 and mGluR5 in the BLA of the CFA group increased significantly (Fig. 3A-B; Fig. 4A-C; Fig. 5A-C; all $P<0.05$). After treatment with different doses of volatile oil, the protein levels of NMDAR1, NMDAR2A, NMDAR2B, mGluR1 and mGluR5 in the BLA showed an overall downward trend when compared to the CFA group and CFA + 0.4 mL/10 g b.w. volatile oil group has the most significant effect, the effect of AGR is dose dependent. There was no significant difference between the CFA group and the CFA + 0.1 mL/10 g b.w. volatile oil group (Fig. 3A-B; Fig. 4A-C; Fig. 5A-C; all $P<0.05$). Compared with the CFA group, the expressions of NMDAR1, NMDAR2A, NMDAR2B, mGluR1 and mGluR5 were significantly decreased in the CFA + 0.2 mL/10 g b.w. volatile oil group and the CFA + 0.4 mL/10 g b.w. volatile oil group (Fig. 3A-B; Fig. 4A-C; Fig. 5A-C; all $P<0.05$).
Fig. 4 Volatile oil of Acori Graminei Rhizoma alleviates the neurotoxic effects of NMDAR2.

A-C. The effect of volatile oil of Acori Graminei Rhizoma on the expression of NMDAR2A and NMDAR2B in the BLA of CFA-injected rats as determined by western blot. \( n=6 \); D-G. The effect of volatile oil of Acori Graminei Rhizoma on the expression of NMDAR2A and NMDAR2B in the BLA of CFA-injected rats as determined by immunofluorescence. \( n=6 \). \( *P<0.05 \), \( **P<0.01 \).
Fig. 5  Volatile oil of Acori Graminei Rhizoma alleviates the neurotoxic effects of mGlur1/5.  
A-C. The effect of volatile oil of Acori Graminei Rhizoma on the expression of mGlur1 and mGlur5 in the BLA of CFA-injected rats as determined by western blot. n=6; D-G. The effect of volatile oil of Acori Graminei Rhizoma on the expression of mGlur1 and mGlur5 in the BLA of CFA-injected rats as determined by immunofluorescence. n=6. *P<0.05, **P<0.01.
3.3 The volatile oil of AGR down-regulates expressions of ERK1/2 and CREB, a downstream signaling pathway of NMDAR

The immunofluorescent results showed that the CFA group showed significant positive expression of ERK1/2 in the BLA of rats compared with the control group (Fig. 6D, 6F; *P*<0.05). While the expression of ERK1/2 in the CFA + 0.2 mL/10 g b.w. volatile oil group and the CFA + 0.4 mL/10 g b.w. volatile oil group were significantly decreased (Fig. 6D, 6F; *P*<0.05). Compared with the control group, the expression level of ERK1/2 was increased in the BLA of the CFA group (Fig. 6A-B; *P*<0.05). Compared with the CFA group, the expression level of ERK1/2 in the BLA showed an overall downward trend in all volatile oil groups, and the CFA + 0.4 mL/10 g b.w. volatile oil group has the most significant decreased (Fig. 6A-B; all *P*<0.05).

In the present study, we investigated the expression of CREB in BLA of rats among different treated groups. The CFA group showed obvious positive expression of CREB in the BLA region of rats when compared to the control group (Fig. 6E, 6G; *P*<0.05). Compared with the CFA group, the expression of CREB were significantly increased in the CFA + 0.2 mL/10 g b.w. volatile oil group and the CFA + 0.4 mL/10 g b.w. the volatile oil group (Fig. 6E, 6G; *P*<0.05). Compared with the control group, the expression of CREB was increased in the BLA of the CFA group (Fig. 6A, 6C; *P*<0.05). The expression of CREB was the most decreased in the CFA + 0.4 mL/10 g b.w. volatile oil of AGR group (Fig. 6A, 6C; *P*<0.05).

3.4 NMDAR inhibitor Ifenprodil can reversed mechanical hyperalgesia and place escape/avoidance

Our results showed that the CFA rats had a lower mechanical pain withdrawal threshold on the 3rd day after surgery when compared to the control group (Fig. 7A-B; *P*<0.05, *P*<0.01). The contralateral mechanical pain withdrawal thresholds were significantly lower on the 5th, 7th, and 14th day after the operation (Fig. 7A, *P*<0.05). The mechanical alldonias of the ipsilateral hind paw of rats in the CFA group were significantly reduced on the 1, 3, 5, 7 and 14 days after surgery (Fig. 7B; *P*<0.05) and the time spent in the light chamber was significantly increased in the CFA group (Fig. 7C; *P*<0.05). Compared with the Ifenprodil group and the NMDA group, single targeted administration of Ifenprodil group to the BLA could reduce ipsilateral mechanical allodynia (Fig. 7B; *P*<0.05), but the time spent in the light chamber was reduced (Fig. 7C; *P*<0.05). These data indicated that Ifenprodil could prevent and reverse CFA-induced mechanical allodynia and avoid mechanical stimulation of painful hind paw of rats.

3.5 NMDAR inhibitor Ifenprodil initiates the expression of mGlur1/5

To determine whether the NMDAR inhibitor initiates the expression of mGlur1/5, we tested mGlur1/5 in the BLA of rats. Compared with the control group, the expression of mGlur1/5 in the BLA of the CFA group were increased (Fig. 8D-G; all *P*<0.01). The mGlur1/5 of positive expression in the NMDA group were significantly increased than those in the CFA + NS group (Fig. 8D-G; all *P*<0.01). Compared with the NMDA group, the positive expressions of mGlur1/5 in the CFA + NS group and Ifenprodil group were significantly reduced (Fig. 8D-G; all *P*<0.01). Compared with the CFA + NS group, the positive expression of mGlur1/5 in the Ifenprodil group showed significant reduced (Fig. 8D-G; *P*<0.01). The data of western blot further confirmed the immunofluorescent results obtained above. Compared with the control group, the expression of mGlur1/5 in the BLA of the CFA group increased significantly (Fig. 8A-C; all *P*<0.05). Compared with the CFA + NS group, the expression of mGlur1/5 in the NMDA group was increased (Fig. 8A-C; all *P*<0.05), while the expression level of mGlur1/5 in the Ifenprodil group was decreased.
Fig. 6 The volatile oil of Acori Graminei Rhizoma down-regulates the expression of ERK1/2 and CREB in the downstream signaling pathway of NMDAR.

A-C. The effect of Volatile oil of Acori Graminei Rhizoma on the expression of ERK1/2 and CREB in the BLA of CFA-injected rats as determined by western blot. n=6; D-G. The effect of Volatile oil of Acori Graminei Rhizoma on the expression of ERK1/2 and CREB in the BLA of CFA-injected rats as determined by immunofluorescence. n=6. *P<0.05, **P<0.01.
Fig. 7 NMDAR inhibitors could reverse mechanical hyperalgesia and position avoidance.
A-C. The effect of NMDA and Ifenprodil on CFA-induced pain and emotional behavior. A. Contralateral mechanical pain withdrawal thresholds; B. Ipsilateral mechanical pain withdrawal thresholds; C. Percent of time within light side of chamber. n=6. *P<0.05, **P<0.01 in figure A, B: CFA group vs control group. ***P<0.01 in figure C: CFA group vs control group. ""P<0.01 in figure B, C: Ifenprodil group vs NMDA group.

3.6 NMDAR inhibitor Ifenprodil initiates the expressions of ERK1/2, CREB, and c-Fos

The immunofluorescent results showed increased positive expressions of ERK1/2, CREB and c-Fos in the BLA of the CFA group when compared to the control group (Fig. 9D-G; Fig. 10C-D; all P<0.05). Furthermore, the positive expressions of ERK1/2, CREB and c-Fos in the NMDA group were significantly increased than those in the CFA + NS group and the Ifenprodil group (Fig. 9D-G; Fig. 10C-D; all P<0.05).

Compared with the CFA + NS group, the positive expressions of ERK1/2, CREB and c-Fos were decreased in the Ifenprodil group (Fig. 9D-G; Fig. 10C-D; all P<0.05). Western blot analysis showed that the expressions of ERK1/2, CREB and c-Fos were significantly increased in the BLA of the CFA group and the CFA + NS group compared to the control group (Fig. 9A-C; Fig. 10A-B; all P<0.05). Compared with the CFA group, the expressions of ERK1/2, CREB and c-Fos were decreased in the Ifenprodil group (Fig. 9A-C; Fig. 10A-B; all P<0.05). Compared with the NMDA group, the expressions of ERK1/2, CREB and c-Fos in the BLA of the Ifenprodil group were significantly reduced (Fig. 9A-C; Fig. 10A-B; all P<0.05).
Fig. 8  NMDAR inhibitor initiates the expression of mGlur1/5 which is coordinated by NMDAR.
A-C. The effect of NMDA and Ifenprodil on the expression of mGlur1 and mGlur5 in the BLA of CFA-injected rats as determined by western blot. n=6; D-G. The effect of NMDA and Ifenprodil on the expression of mGlur1 and mGlur5 in the BLA of CFA-injected rats as determined by immunofluorescence. n=6. *P<0.05, **P<0.01.
Fig. 9 NMDAR inhibitor initiates the expression of its downstream signaling pathway’s ERK1/2, CREB.
A-C. The effect of NMDA and Ifenprodil on the expression of ERK1/2 and CREB in the BLA of CFA-injected rats as determined by western blot. n=6; D-G. The effect of NMDA and Ifenprodil on the expression of ERK1/2 and CREB in the BLA of CFA-injected rats as determined by immunofluorescence. n=6. *P<0.05, **P<0.01.
4 Discussion

Our findings demonstrate that the role of AGR can reduce mechanical hyperalgesia and positional avoidance in animals caused by CFA. The expression of NMDAR1, NMDAR2A, NMDAR2B, mGlur1/5, ERK1/2, and CREB was downregulated in an AGR dose dependent manner, NMDAR inhibitor Ifenprodil upregulates the expression of c-Fos, reversing the above phenomenon the expression of NMDAR and related factors in downstream pathways can relieve pain-related aversion induced by inflammatory pain. Research shows that Ifenprodil, an NR2B antagonist, is more effective in pain treatment with fewer side effects[28]. The present study indicated that Ifenprodil, an NR2B antagonist, has an effect on the pathogenesis of pain-related aversion induced by inflammatory pain, which can activate the conduction pathway of mGlur1/5-ERK1/2-CREB-c-Fos. This study also shows that NMDAR and mGlur1/5 have a synergistic effect in the pathogenesis of inflammatory pain-induced pain-related emotions. However, mechanisms of
the volatile oil of AGR and NR2B antagonist on therapeutic effects of pain-related emotions induced by inflammatory pain need further investigation.

The amygdala, as the almond-shaped marginal structure in the temporal lobe of the brain, plays an important role in avoidance behavior of threatening stimuli and integration of pain and pain-related negative emotions\[29-31\]. Any dysfunction and/or structural changes in the amygdala may have substantial consequences on emotion and pain memory consolidation as well as pain aversion behavior\[6,32\]. The amygdala is composed of several functionally distinct nuclei, including the lateral (LA), basolateral (BLA), and central (CeA) nuclei. The BLA is particularly important for integrating emotional processing and sensory\[33\]. The above findings suggest that BLA is associated with pain-related negative emotional responses and memory formation. Therefore, clarifying the emotional response related to pain and the formation process of pain memory is helpful to understand the integration mechanism of pain signals in the amygdala. CFA is a conventional model for studying chronic inflammatory pain in rodents\[34-35\]. In this study, rats had obvious redness and swelling and paw lifting, contraction, paw licking and vocalization the next day after injection of CFA.

Glutamate is a neurotransmitter mainly responsible for excitatory nerve conduction in the central nervous system. It is divided into ionotropic glutamate receptors and metabotropic glutamate receptors\[36\]. NMDAR is one of three ligand-gated ion channels activated by the excitatory transmitter glutamate. The specific antagonist of NR2B had a greater pain relief effect than other NMDA receptor antagonists\[37-38\]. Some studies have shown that activation of ERK/CREB pathway can inhibit inflammatory pain induced by CFA in rats\[39\]. Metabotropic glutamatergic receptor (mGluR) 1 and mGluR5, members of group I mGluRs, play crucial roles in central sensitization and chronic pain\[40\]. The mGluR is expressed in the central nervous system and participates in the regulation of neuronal function and synaptic plasticity through the interaction with NMDA receptor\[41\]. It had been reported that mGluR1 and mGluR5 mainly increased the activity of NMDA receptors, and there was a risk of excitotoxicity\[28\]. The mGluR5 activation could increase the phosphorylation level of downstream ERK1/2. In addition, studies have found that, cytohesin-2 conditional knockout mice exhibited reduced mechanical allodynia and ERK1/2 activation following the pharmacological activation of spinal mGluR1/5 with 3,5-dihydroxyphenylglycine (DHPG)\[42\].

ERK is a member of the MAPK family, which is activated by neuronal activity and participates in neuronal plasticity\[43\]. Recently, some studies have revealed that the activation of ERK in the brain region could lead to the production of inflammatory mediators after inflammation or nerve injury\[44\]. It has been reported that formalin-induced inflammatory pain significantly up-regulated the expression of ERK in the cingulate cortex of the brain, and ERK plays a key role in thermal hyperalgesia and tactile allodynia\[45-46\]. cAMP response element binding protein (CREB), which can be activated by N-cadherin, is involved in the induction and maintenance of LTP18 and contributes to pain modulation in the spinal cord during the transition from acute to chronic pain\[47\]. The c-Fos family is used as a marker of neuronal activity and can be regulated by stress\[48\]. Studies have shown that c-Fos in the BLA was up-regulated in mice with nerve injury showing anxiety-like behaviors\[49\].

In this study, firstly, the changes in pain-related, emotional behavior and related emotions were observed in CFA model rats through pain-related and emotional behavioral tests, and further detected the pain-related and emotional changes in rats treated with volatile oil from AGR. Finally, immunofluorescence and Western blot methods were used to detect the correlation between the
expression levels of NMDAR and its downstream factors in the BLA and related emotional behavioral changes. Furthermore, in order to directly regulate the expression of NMDAR, NMDA agonist and NMDAR2B antagonist Ifenprodil were respectively injected into the BLA using brain stereotaxic apparatus, and then changes of affective-motivational pain responses behavior in rats were observed. Subsequently, immunofluorescence and Western blot methods were used to verify the synergistic mGlur1/5 and some downstream factors, to further clarify the mechanism of NMDAR in the BLA involved in the formation of pain-related emotions induced by inflammatory pain. Therefore, in this study, we aimed to explore whether the volatile oil of AGR could alleviate the negative emotions induced by the CFA pain model by activating the ERK/c-Fos pathway. Based on the above researches, our results showed that the expression of NMDARs in the BLA and downstream pathways ERK1/2-CREB-c-Fos, which play the key roles in the formation of pain-related emotions. Affective-motivational pain responses induced by inflammatory pain were related to the activation of NMDARs/mGlur1/5 and downstream pathway ERK1/2-CREB-c-Fos. After treatment with the volatile oil of AGR, the expressions of the above factors in this pathway could be inhibited, thereby affective-motivational pain responses induced by inflammatory pain of animals were alleviated. To study whether the relieving effect of the volatile oil of AGR on affective-motivational pain responses induced by chronic inflammatory pain and which is mediated by NMDARs and downstream pathway ERK1/2-CREB-c-Fos. In this study, the NMDAR2B antagonist Ifenprodil was injected into the BLA using brain stereotactic targeting, which could significantly relieve affective-motivational pain responses induced by inflammatory pain and be related to the activation of the downstream conduction pathway of NMDAR. Besides, this study also suggested that NMDARs and mGlur1/5 have a synergistic effect in the pathogenesis of pain-related emotions induced by inflammatory pain. The ERK/c-Fos pathway was activated in the CFA-induced chronic inflammatory pain model, and selective inhibition of NMDAR receptors could markedly reduce the related negative emotions induced by the CFA pain model.

In conclusion, the present study was carried out to explore the possible mechanism for the volatile oil of AGR in alleviating the pain-related emotions induced by inflammatory pain in rats. However, the specific mechanism of the synergy between NMDAR and mGlur1/5 is still unclear, so this study still needs to be further explored. The research may provide a new therapeutic strategy for clinic treatment of affective-motivational pain responses induced by pain and related drug development.

5 Conflicts of interest

The authors have no conflict of interest to declare.

6 Author Contribution Statement

MA Yuxin and LI Shiqi: Designed the study and wrote the paper. LI Shiqi, YANG Cuizhu, ZHANG Runheng and WANG Shuhan: Conducted the data analysis and revised article. YANG Yaqi, LIU Jing, and LI Guoying: Provided technical guidances.

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8 Data availability

Enquiries about data availability should be directed to the authors.

References


