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Research paper

Irisin alleviates chronic constriction injury-induced hyperalgesia and affective disorders in mice through NF-κB and Nrf2 signaling pathways

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ABSTRACT

This research is to explore the impacts of irisin on hyperalgesia and behavioral deficits caused by chronic constriction injury (CCI) and the underlying mechanisms. The CCI mice model was used in this study. The experimental mice were assigned into sham, sham + irisin (3 μg/kg), CCI, CCI + irisin (0.1, 1, and 3 μg/kg), and CCI + irisin (3 μg/kg) + ML385 (30 mg/kg) groups. The results showed that after CCI injury, the mice exhibited hyperalgesia, depression, and anxiety. In addition, the levels of inflammatory cytokines NF-κB, IL-1β, IL-6, TNF-α, and iNOS increased in the mice hippocampus, frontal cortex, and spinal cord. Moreover, oxidative stress relevant factor MDA increased, while GSH and SOD decreased in the mice hippocampus, frontal cortex, and spinal cord. However, irisin treatment ameliorated CCI-induced mechanical allodynia, thermal hyperalgesia, depressive, and anxiety behaviors, and reversed the abnormal expressions of inflammatory and oxidative stress relevant cytokines. Interestingly, these therapeutic effects of irisin were partly abolished by ML385, a specific Nrf2 antagonist. Taken together, irisin may be an effective therapeutic agent for CCI-induced neuralgia and the affective disorders, and the mechanisms may be associated with the anti-neuroinflammation mediated by NF-κB and the anti- oxidative stress function regulated by Nrf2.

1. Introduction

Chronic neuralgia is a disease characterized by oversensitivity to harmless or harmful stimuli (Macone and Otis, 2018), affecting about 20 % of the total population (Mokhtari et al., 2023). This type of neural pain is always accompanied by affective disorders such as depression and anxiety. As reported, up to 80 % of patients with chronic pain suffer from mental health disorders, e.g., depression and anxiety (Armbrecht et al., 2021; Mokhtari et al., 2024). The animal model of neuropathic pain, chronic constriction injury (CCI) model, reproduces the main symptoms of neuropathic pain experienced in humans and represents a fundamental tool for the development of effective drugs (Medeiros et al., 2021). Traditional drugs for chronic neuralgia do not show desired therapeutic effects on the associated affective disorders and have strong adverse reactions (Gradl et al., 2013). Therefore, there are ongoing studies exploring effective and safe medications for neuralgia and the comorbid affective disorders.

Neuroinflammation is considered a primary process involved in the pathogenesis of chronic neuralgia and the comorbid affective disorders (Ferreira-Chamorro et al., 2018; Yang et al., 2022). Previous studies reported that both acute and chronic pain is associated with the modulation of glial cells, as well as neuroinflammation in the prefrontal cortex and hippocampus. Neuroinflammation of the hippocampus is key to the development of depressive and anxious disorders following chronic pain (Tahmineh et al., 2019). The detail mechanism could be that pain hypersensitivity stimulates glial cells to activate multiple signaling pathways to regulate pain management in the spinal cord and secreting inflammatory chemokines and cytokines to promote pain transmission (Tiwari et al., 2014). The characteristic of neuroinflammation is the increase in the levels of inflammatory factors such as chemokines and cytokines, which are widely distributed in local tissues, peripheral nerves, dorsal root ganglia, and central nervous system, thus contributing to the occurrence and maintenance of chronic pain (Ji et al., 2014; B.C. Jiang et al., 2020). Other studies found that chronic

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neuralgia leads to increase in the activity of nuclear factor-kappa B (NF-κB), a crucial transcription factor that controls the expression of many inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS) (Arruri et al., 2017; X. Jiang et al., 2018).

Oxidative stress is also an important pathological mechanism for chronic neuropathy and the associated emotional disorders (Mokhtari et al., 2023). Chronic neuralgia leads to the occurrence of oxidative stress, which is characterized by the atypical generation of glutathione (GSH), malondialdehyde (MDA), and superoxide dismutase (SOD) (Arruri et al., 2017). The mechanism underlying this process involves the antioxidative signaling pathway mediated by the nuclear factor erythroid 2-related factor 2 (Nrf2) (Haghani et al., 2022; Kaur et al., 2016). Hence, therapies aiming at reducing oxidative stress and inflammation have the potential to be effective treatment approaches in preventing chronic neuralgia and the associated mood disorders.

For the investigation of treatments for chronic neuralgia, multiple studies proved that herbal medicines present promising efficacy. For example, a study performed by Chu et al. demonstrated that loganin improves CCI-induced neuroinflammation and pain behavior by down-regulating TNF-α/IL-1β-dependent NF-κB activation (Chu et al., 2020). In addition, Tahmineh et al.'s study found the potency of luteolin in the improvement of chronic pain-induced anxiety- and depressive-like symptoms (Mokhtari et al., 2023). However, the potential effects of irisin, a skeletal muscle-derived myokine secreted after physical exercise (Boström et al., 2012), on chronic neuralgia is rarely studied.

In the last decade, a few studies have indicated that irisin may possess therapeutic function on chronic pain and the associated neurological disorders. For example, a previous study had reported the analgesic effect of irisin on acute pain (Boström et al., 2012). In addition, evidence proved that the circulating irisin possesses antioxidant and anti-inflammatory features and can be used to prevent and treat neurological disorders (S. Liu et al., 2022). However, the therapeutic value and relevant mechanisms of irisin on chronic pain are unknown, and the neuronal mechanisms underlying the effects of irisin on neuralgia-induced affective disorders is to be disclosed. Therefore, we try to explore the potential therapeutic effects of irisin on chronic neuralgia and the relevant affective disorders. Besides, whether the function of irisin is related to its effects on inflammation and oxidative stress is also investigated. As the transmission of chronic pain is associated with the pain management in the spinal cord and neuroinflammation in the prefrontal cortex and hippocampus (Tahmineh et al., 2019; Tiwari et al., 2014), we selected hippocampal, frontal cortex and spinal cord as preparations for the biochemical analysis to explore the underlying mechanism. To the best of our knowledge, this is the first study regarding irisin's potential effects on chronic neuralgia involving anti-inflammation and anti-oxidative stress mechanisms.

2. Materials and Methods

2.1. Animals

The animals used in this study were 4–6-week-old male ICR mice weighing 20–22 g. They were obtained from Hangzhou Medical Laboratory Animal Center (Hangzhou, China). Before the experiments, the mice were housed in cages under a controlled environment (temperature: 21–27℃; humidity: 50±10 %). All the experimental protocols were approved by the Animal Care and Use Committee of Zhejiang Pharmaceutical University and conducted in accordance with the regulations set by the Committee.

2.2. Chronic constriction injury (CCI) model

Mice were anesthetized with ketamine (concentration: 75 mg/kg) and then fixed on the operating table. Bilateral sciatic nerves of the mice were exposed with glass tweezers, and three loose knots on the sciatic nerve of each side were tied using an absorbable catgut suture. The muscles and epidermis were then sewn (X. (Jiang et al., 2018). After the surgery, the mice were treated with penicillin (concentration: 40000 U/kg) for 3 days to avoid infection.

2.3. Animal grouping and experimental assignment

The mice were randomly assigned into the following groups: sham group, sham + irisin (3 μg/kg, intravenous (i.v.)) group, CCI group, CCI + irisin (0.1, 1, and 3 μg/kg, i.v.) group, and CCI + irisin (3 μg/kg, i.v.) + ML385 (Nrf2 antagonist) (30 mg/kg, intraperitoneal (i.p.)) group. Each group included 16 mice. Doses of irisin were selected based on previous studies (Aydoğdu et al., 2019; X. Jiang et al., 2021). The irisin was administrated via injecting the irisin solution into one of the lateral veins of the mouse tail.

The behavior tests in this study include locomotor activity, pain-related behavior tests (thermal hyperalgesia test and mechanical hyperalgesia test), depression tests (sucrose preference test and tail suspension test), and anxiety tests (marble burying test and elevated plus maze test). From the 7th day to the 34th day after CCI surgery, freshly prepared recombinant irisin (Rocky Hill, NJ, USA) or vehicle (non-irisin treatment groups) was administered at 8:00AM on each day. ML385 was administrated daily from the 28th day to the 34th day. Pain-related behavior tests including thermal hyperalgesia test (THT) and mechanical hyperalgesia test (MHT) were performed on day 7, 11, 15, 19, 23, 27, 28, 31, and 34 for all the mice in each group. Locomotor activity assessment, depression tests including sucrose preference test (SPT) and tail suspension test (TST), and anxiety tests including marble burying test (MBT) and elevated plus maze test (EPMT) were performed on day 7, 14, 21, 28, and 34. The pain-related behavior tests and locomotor activity assessment were performed for all the animals in each group. While the depression tests were performed in 8 mice and the anxiety tests were performed in the remaining 8 mice in each group. When all the behavior tests were completed, all the mice were anesthetized with diethyl ether, and 2.5 μL cerebrospinal fluid (CSF) was collected. Finally, the mice were decapitated, and the serum and tissues including hippocampus, frontal cortex, and spinal cord were collected for biological tests (refer to Fig. 1 for experimental schedule).

2.4. Behavioral tests

2.4.1. Thermal hyperalgesia test (THT)

Before testing, each animal was placed in the test box for 30 min. The bottom of the box was made of glass. We then used an infrared heat source to stimulate the paws of mice through the glass. This heat source can provide and maintain a temperature at 46 °C ± 0.5 °C. The data on latency of each animal were collected at 3-min intervals, and an average of triplicate readings was taken to eliminate any discrepancies (Hargreaves et al., 1988).

2.4.2. Mechanical hyperalgesia test (MHT)

Each mouse was put into the testing cage for a duration of 20 min prior to the test. The bottom of the cage was a mesh, the perforation of which was within 1 cm^2 area. The paws of each animal were stimulated with a series of Von-Frey filaments that are equivalent to 0.16, 0.4, 0.6, 1, 1.4, 2, 4, 6, 8, and 10 g forces. The data of pain threshold were recorded as the average of triplicate readings to eliminate any discrepancies and expressed as grams (Campana and Rimondini, 2021).

2.4.3. Sucrose preference test (SPT)

Each mouse was given both 1 % sucrose water solution and regular drinking water for 24 hours prior to the CCI surgery. After the surgery, each mouse was given both solutions for 1 hour. The ratio of the amount of sucrose water consumed to the total amount of water consumed was recorded (Yu et al., 2013).

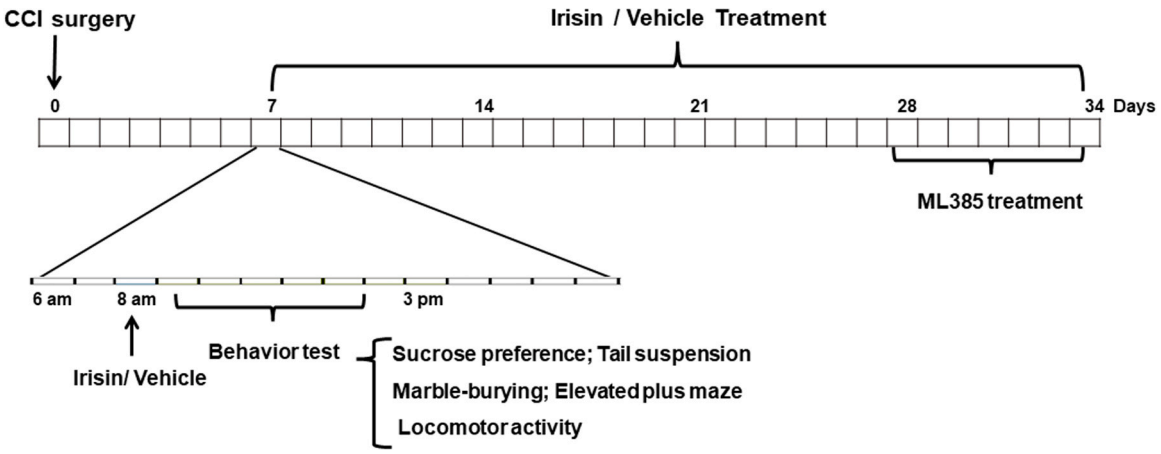

Fig. 1. Experimental schedule. Mice received administration of irisin or vehicle daily from the 7th day to the 34th day after CCI surgery; ML385 was co-administered with irisin at last week. Behavioral tests were performed from 9 am to 1 pm. Behavior tests for locomotor activity, depression, and anxiety were performed on day 7, 14, 21, 28, and 34. Pain-related behavior tests (THT and MHT) were performed on day 7, 11, 15, 19, 23, 27, 28, 31, and 34.

2.4.4. Locomotor activity

Each mouse was positioned within the testing chamber that was connected to the behavioral testing apparatus. The locomotor activity of each mouse was recorded for a duration of 10 min. Subsequent to each test, the chamber was sanitized using alcohol (X. Jiang et al., 2016).

2.4.5. Tail suspension test (TST)

TST was conducted in accordance with a previously established protocol. Each mouse was suspended with the tip of its tail 50 cm from the ground for a duration of 6 minutes. The duration of immobility in the last 4 min was calculated as the testing result (X. Jiang et al., 2016).

2.4.6. Marble burying test (MBT)

Each test cage contained 9 glass marbles. The marbles were equidistantly placed on corn cobs of 5 cm depth. During a 10-min experimental period, the quantity of marbles buried by each mouse was recorded (Trexler et al., 2018).

2.4.7. Elevated plus maze test (EPMT)

Each mouse was initially placed at the central region of an elevated plus maze. The time spent in the closed and open arms were respectively recorded during a 10-min experimental period (X. Jiang et al., 2020).

2.5. Biochemical analysis

After behavior tests (34th day after CCI), the mice were anesthetized with diethyl ether, and 2.5 μL cerebrospinal fluid (CSF) was collected from each mouse by aspiration. Subsequently, the mice were decapitated, and the tissues including hippocampus, frontal cortex, and spinal cord were dissected; serum was also obtained from the collected blood.

2.5.1. Quantitative real-time polymerase chain reaction (PCR)

The levels of iNOS mRNA in the hippocampus, frontal cortex, and spinal cord were estimated by PCR. The tissue samples were processed using the RNA kit provided by Bio-Rad Laboratories. The primer sequences for the target RNAs are presented in Table 1.

2.5.2. Quantification of oxidative stress markers

The levels of MDA, GSH, and SOD in the hippocampus, frontal cortex, and spinal cord were assessed using a commercially available kit provided by Thermo Scientific. The SOD, MDA, and GSH levels were expressed as U/mg protein, nmol/mg protein, and nmol/mg protein, respectively.

2.5.3. ELISA

The expression levels of IL-1β, IL-6, TNF-α, and NF-κBp65 in the hippocampus, frontal cortex, and spinal cord were quantified using the corresponding ELISA kits (Thermo Scientific, USA). The expression levels of iNOS and Nrf2 were measured using ELISA kits from Abcam, USA and Cusabio, China, respectively. The optical density (OD) values IL-1β, IL-6, TNF-α, Nrf2 and iNOS were measured at a wavelength of 450 nm. The OD value of NF-κBp65 was determined at a wavelength of 405 nm. Irisin levels in the serum, CSF, and spinal cord were measured using an ELISA kit from Phoenix Pharmaceuticals, Burlingame, CA, USA. Intra-assay (within days) and inter-assay (between days) values were 4–6 % and 8–10 %, respectively. The OD value of irisin was determined a wavelength of 450 nm. Sample absorbance was read by an ELX 800 ELISA reader.

2.6. Statistical analysis

Two-way analysis of variance (ANOVA) and One-way ANOVA were used to analyze the differences between groups. For two-way ANOVA, the number of days was regarded as factor A, the procedure (CCI or not) and the treatments (irisin or not) were regarded as factor B. The data of behavioral tests, including THT, MHT locomotor activity, SPT, TST, MBT and EPMT in Figs. 2–3 were analyzed using repeated measures two-way ANOVA, and the other data were analyzed using one-way ANOVA. Dunnett's test was used for post hoc analysis. The data are presented as mean ± standard error of the mean (SEM). p value below 0.05 was considered statistically significant.

Table 1
Primer sequences for iNOS and β-actin.

Target	Forward (5′-3′)	Reverse (5′-3′)
iNOS	CCTCCTCCACCCTACCAAGT	CACCCAAAGTGCTTCAGTCA
β-actin	TGGAATCCTGTGGCATCCATGAAAC	AAAACGCAGCTCAGTAACAGTCCG

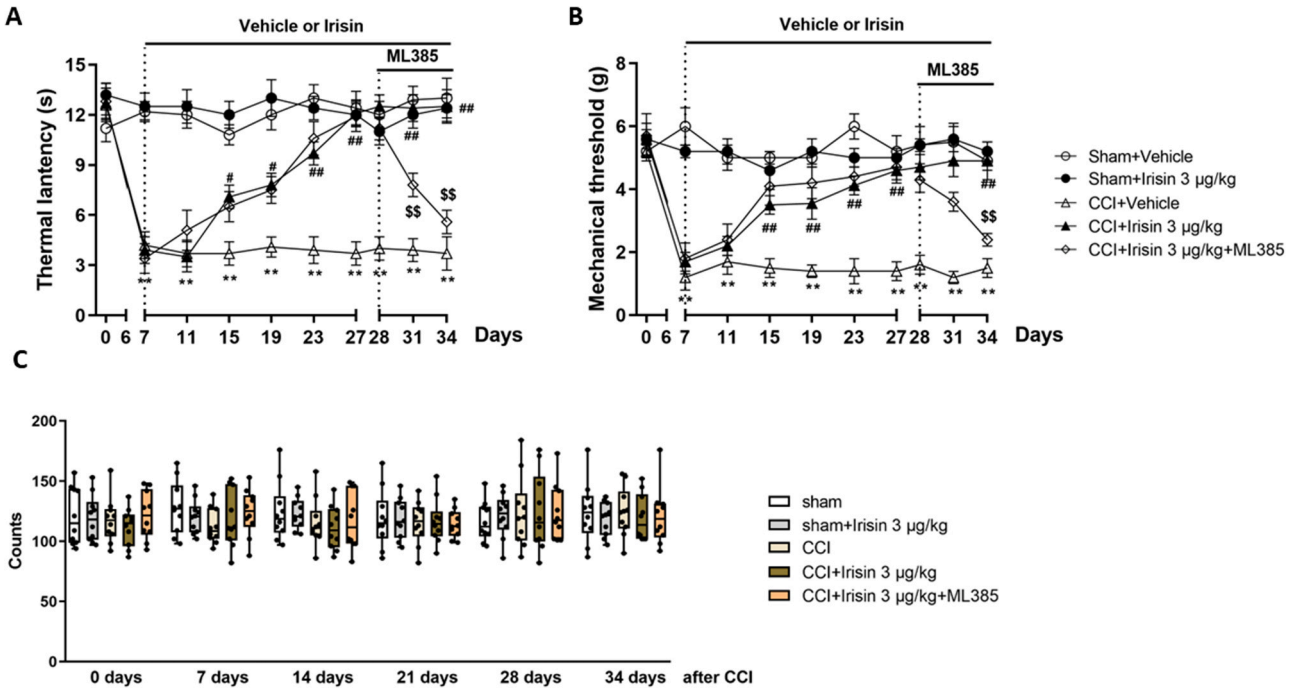

Fig. 2. Effects of irisin (3 μg/kg, i.v.) on thermal hyperalgesia (A), mechanical hyperalgesia (B), and locomotor activity (C) in mice. n = 16 per group. ** $p < 0.01$ versus sham; $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ versus CCI; $^{\$\$}$ $p < 0.01$ versus irisin (3 μg/kg).

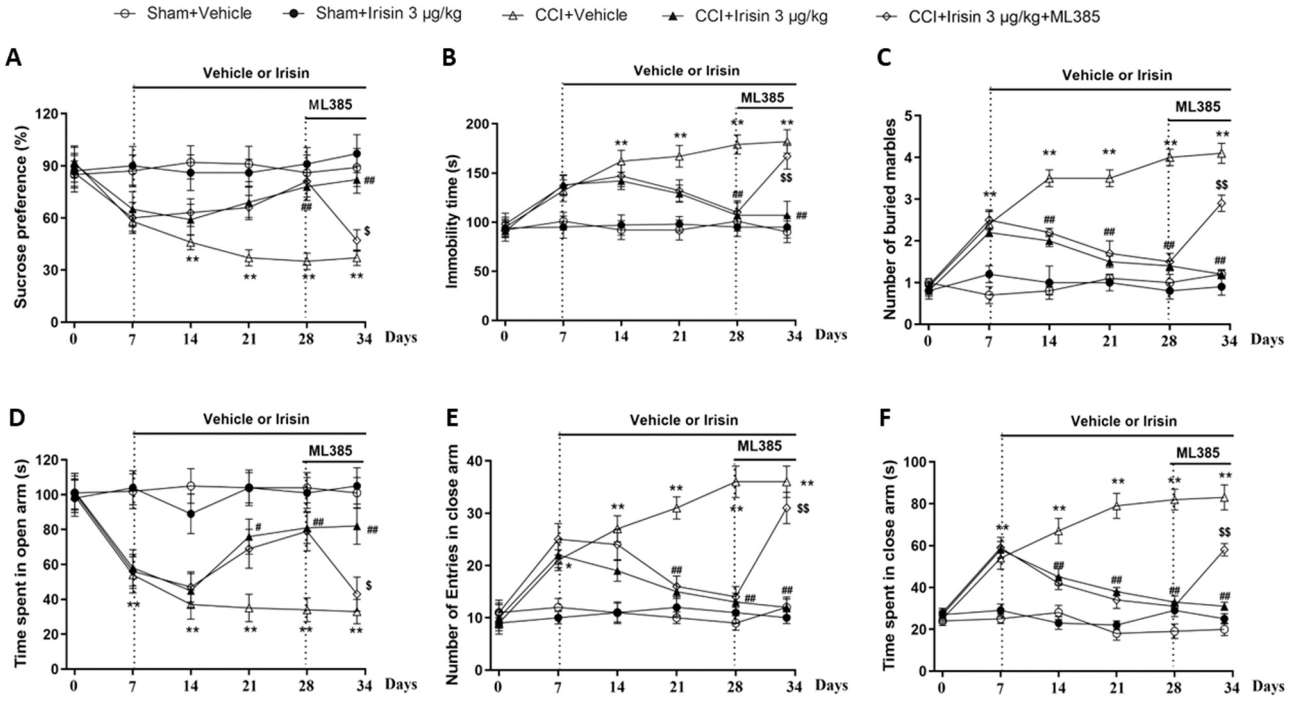

Fig. 3. Effects of irisin (3 μg/kg, i.v.) on sucrose preference test (A), tail suspension test (B), marble-burying test (C), and elevated plus maze test (D, E, F) in mice. n = 8 per group. *p <0.05 and **p < 0.01 versus sham; $^{\#}p$ < 0.05 and $^{\#\#}p$ < 0·01 versus CCI; $^{\$}p$ < 0.05 and $^{\$\$}p$ < 0.01 versus irisin (3 μg/kg).

3. Results

3.1. Irisin reduced hyperalgesia in the CCI mice

As shown in Fig. 2, the mice in the CCI group at the 34th day exhibited remarkable mechanical allodynia and thermal hyperalgesia as compared with the sham group. Irisin treatment at a dose of 3 μg/kg relieved the mechanical allodynia [F (4, 350) = 17.9 for factor B, p < 0.01, Fig. 2**A**] and thermal hyperalgesia [F (4, 350) = 12.2 for factor B, p < 0.01, Fig. 2**B**], and the maximin effects were seen after 4 weeks of treatment post-surgery. However, ML385 co-treatment from the 28th day to the 34th day gradually neutralized the analgesic effects of irisin. The effects of irisin at doses of 0.1 and 1 μg/kg on CCI-induced hyperalgesia are summarized in Supplementary Fig. 1. Data in Fig. 2**C** implies that neither irisin treatment nor CCI or sham surgery influenced the locomotor activity of the mice.

3.2. Irisin ameliorated the depressive-like behaviors in the CCI mice

As shown in Fig. 3**A**, the mice in the CCI group at the 34th day exhibited a loss of preference for sucrose water after CCI surgery [F (4, 210) = 20.4 for factor B, $p < 0.01$]. However, chronic treatment with irisin significantly ameliorated this phenomenon ($p < 0.01$). In the TST (Fig. 3**B**), the CCI group mice showed a significant increase in immobility time [F (4, 210) = 30.4 for factor B, $p < 0.01$], Whereas irisin treatment alleviated this adverse symptom ($p < 0.01$). Interestingly, the combined administration of irisin and ML385 reversed the effects of irisin on sucrose preference in the SPT ($p < 0.05$) and immobility in the TST ($p < 0.01$). [sucrose preference test: F (5, 210) = 3.4, p <0.01 for factor A, and F (4, 210) = 20.4, p <0.01 for factor B; tail suspension test: F (5, 210) = 7.2, p <0.001 for factor A, and F (4, 210) = 30.4, P<0.01 for factor B; marble-burying test: F (5, 210) = 23.0, p <0.01 for factor A, and F (4, 210) = 125.7, p <0.01 for factor B; time spent in the open arm: F (5, 210) = 7.6, p <0.01 for factor A, and F (4, 210) = 33.9, p <0.01 for factor B; number of entries in close arm: F (5, 210) = 13.2, p <0.01 for factor A, and F (4, 210) = 60.1, p <0.01 for factor B; time spent in the close arm: F (5, 210)=17.1, p <0.01 for factor A, and F (4, 210) = 130.8, p <0.01 for factor B].

3.3. Irisin ameliorated the anxiety-like behaviors in the CCI mice

As shown in Fig. 3**C**, in the MBT, the CCI group showed a higher number of marbles buried by the mice as compared with the sham group [F (4, 210) = 125.7 for factor B, $p < 0.01$]. However, chronic irisin treatment at 3 μg/kg prevented this increase ($p < 0.01$). In the EPMT, the mice in the CCI group spent significantly less time exploring in the open arms and more time in the closed arms [time spent in the open arm: F (4, 210) = 33.9 for factor B, p <0.01, Fig. 3**D**; time spent in the close arm: F (4, 210) = 130.8 for factor B, p <0.01, Fig. 3**E**]. The number of entries into the closed arms is higher in the CCI group [F (4, 210) = 60.1 for factor B, $p < 0.01$, Fig. 3**F**]. However, treatment with irisin (3 μg/kg) relieved the anxiety-like behavior caused by CCI. Co-treatment with ML385 reversed the anti-anxiety effects of irisin in both MBT and EPMT. The effects of irisin at lower doses (0.1 and 1 μg/kg) on CCI-induced depression and anxiety behaviors are summarized in Supplementary Fig. 2.

3.4. CCI decreased irisin expression levels in the serum, CSF, and spinal cord of the mice

Data of irisin expression are assessed by one-way ANOVA, and statistically significant differences were observed in data between groups in Fig. 4**A-C** [F (4, 25) =14.38, p < 0.01 for Fig. 4**A**; F(4,25)=9.4, p < 0.01 for Fig. 4**B**; F(4,25)=8.3, p < 0.01 for Fig. 4**C**]. The mice in the CCI group showed significantly decreased irisin levels in the serum ($p < 0.01$), CSF ($p < 0.01$), and spinal cord ($p < 0.05$) as compared with the sham group. Treatment with exogenous irisin at 3 μg/kg increased the irisin levels in these tissues.

3.5. Irisin increased nuclear Nrf2 activity and decreased NF-κBp65 expression in the brain and spinal cord of the CCI mice

Significant differences of NF-κBp65 expression were observed between groups in Fig. 5**A-5 C** [F(4,25)=13.98, $p < 0.01$ for Fig. 5**A**; F (4,25)=11.83, $p < 0.01$ for Fig. 5**B**; F(4,25)=20.21, $p < 0.01$ for Fig. 5**C**]. The overexpression of NF-κBp65 was observed in the hippocampus, frontal cortex, and spinal cord of the mice in the CCI group ($p < 0.01$, Fig. 5**A-5 C**). Irisin at 3 μg/kg significantly inhibited the overexpression of NF-κBp65 in these regions ($ps < 0.01$); the inhibitory effect of irisin was abolished by co-treatment with ML385.

Significant differences of Nrf2 expression were observed between groups in Fig. 5**D-5 F** [F(4,25)=17.47, $p < 0.01$ for Fig. 5**D**; F(4,25)= 15.33, $p < 0.01$ for Fig. 5**E**; F(4,25)=7.10, $p < 0.01$ for **Fig, 5 F**]. The expression of nuclear Nrf2 in the hippocampus, frontal cortex, and spinal cord were notably reduced ($p < 0.01$, Fig. 5**D-5 F**). Irisin treatment significantly reversed CCI-induced reduction in Nrf2 expression in these neural regions. While the concurrent administration of ML385 mitigated the impact of irisin on the expression of nuclear Nrf2 in these tissues.

3.6. Irisin treatment improved Nrf-2-mediated oxidative stress markers (MDA, GSH, and SOD) in the CCI mice

As shown in Fig. 6, the CCI mice have significantly higher MDA levels [F(4,25)=17.57, $p < 0.01$ for Fig. 6**A**; F(4,25)=12.11, $p < 0.01$ for Fig. 6**B**; F(4,25)=13.95, p < 0.01 for Fig. 6**C**] and significantly lower GSH and SOD activities [For GSH, F(4,25)=11.65, p < 0.01 for Fig. 6**D**; F (4,25)=8.86, $p < 0.01$ for Fig. 6**E**; F(4,25)=9.90, $p < 0.01$ for Fig. 6**F**] [For SOD, F(4,25)=16.72, $p < 0.01$ for Fig. 6**G**; F(4,25)=12.86, $p < 0.01$ for Fig. 6**H**; F(4,25)=14.65, $p < 0.01$ for Fig. 6**I**] in the hippocampus, cerebral cortex, and spinal cord as compared with the mice in the sham group. Irisin treatment alleviated CCI-induced oxidative stress, and this effect was abolished by co-treatment with ML385.

3.7. Irisin treatment affected NF-κB-mediated inflammatory factors (IL-1β, IL-6, and TNF-α) and the neurotoxic mediator iNOS in the CCI mice

The CCI mice have higher iNOS mRNA levels in the hippocampus, frontal cortex, and spinal cord [F(4,25)=16.50, $p < 0.01$ for Fig. 7**A**; F (4,25)=20.30, $p < 0.01$ for Fig. 7**B**; F(4,25)=19.92, $p < 0.01$ for Fig. 7**C**]. Besides, the iNOS protein levels were also increased in these neural regions [F(4,25)=12.99, $p < 0.01$ for Fig. 7**D**; F(4,25)=15.97, $p < 0.01$ for Fig. 7**E**; F(4,25)=14.82, $p < 0.01$ for Fig. 7**F**]. However, irisin treatment decreased the overexpression of iNOS mRNA and protein levels induced by CCI. After the CCI surgery, the expression levels of IL-1β, IL-6, and TNF-α were drastically increased in the hippocampus [F(4,35)=7.72, p

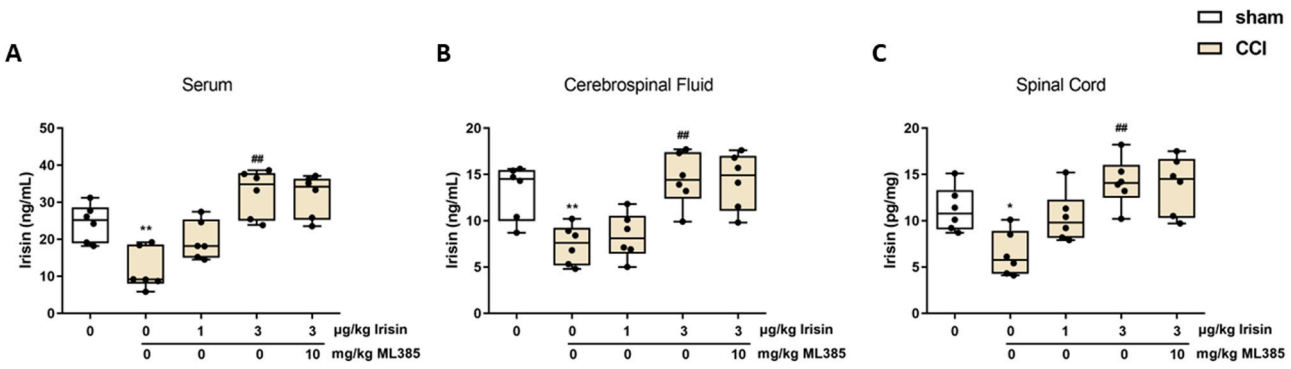

Fig. 4. Expressions of irisin in the serum (A), cerebrospinal fluid (B), and spinal cord (C) of sham/CCI mice. n = 6 per group. *p <0.05 and **$p < 0.01$ versus sham; ##$p < 0.01$ versus CCI; $$$p < 0.01$ versus irisin (3 μg/kg). CSF: cerebrospinal fluid.

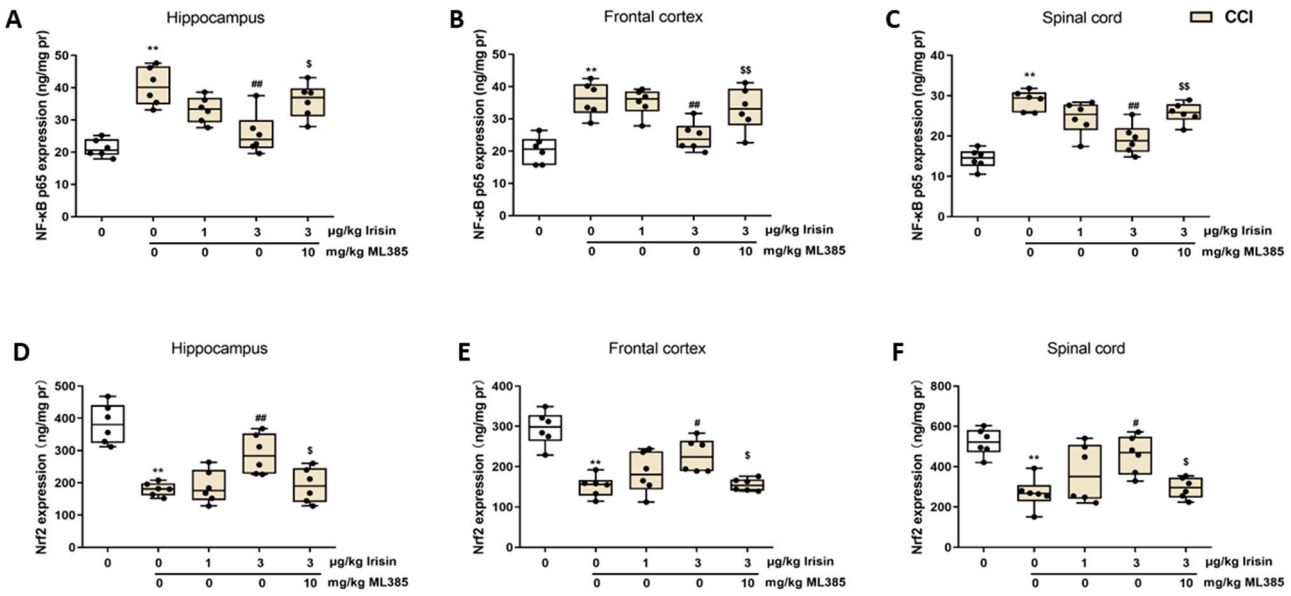

Fig. 5. Effects of irisin on the NF-κB p65 expression in the hippocampus (A), frontal cortex (B), and spinal cord (C) in CCI mice. Effects of irisin on Nrf2 expressions in the hippocampus (D), frontal cortex (E), and spinal cord (F) in CCI mice. Results are expressed as mean ± SEM from 6 mice. $^{**}p < 0.01$ versus sham; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus CCI; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ versus irisin (3 μg/kg). Iri-1:1 μg/kg irisin, Iri-3:3 μg/kg irisin.

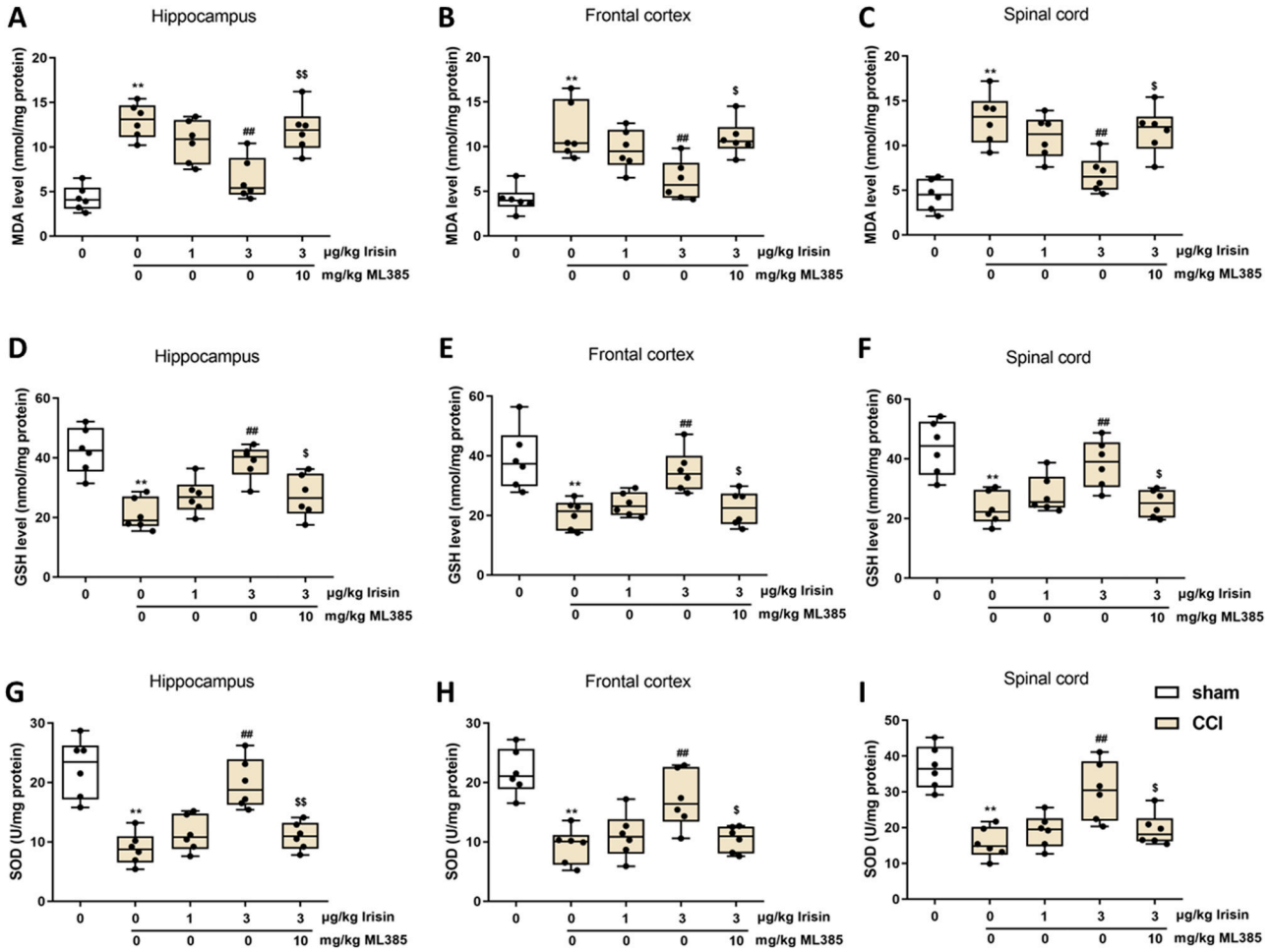

Fig. 6. Effects of irisin on the production of MDA (A-C), GSH (D-F) and SOD levels (G-I) in the hippocampus, cerebral cortex, and spinal cord of CCI mice. Values are expressed as mean ± S.E.M. with 6 mice in each group. $^{**}p < 0.01$ versus sham; $^{\#\#}p < 0.01$ versus CCI; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ versus irisin (3 μg/kg).

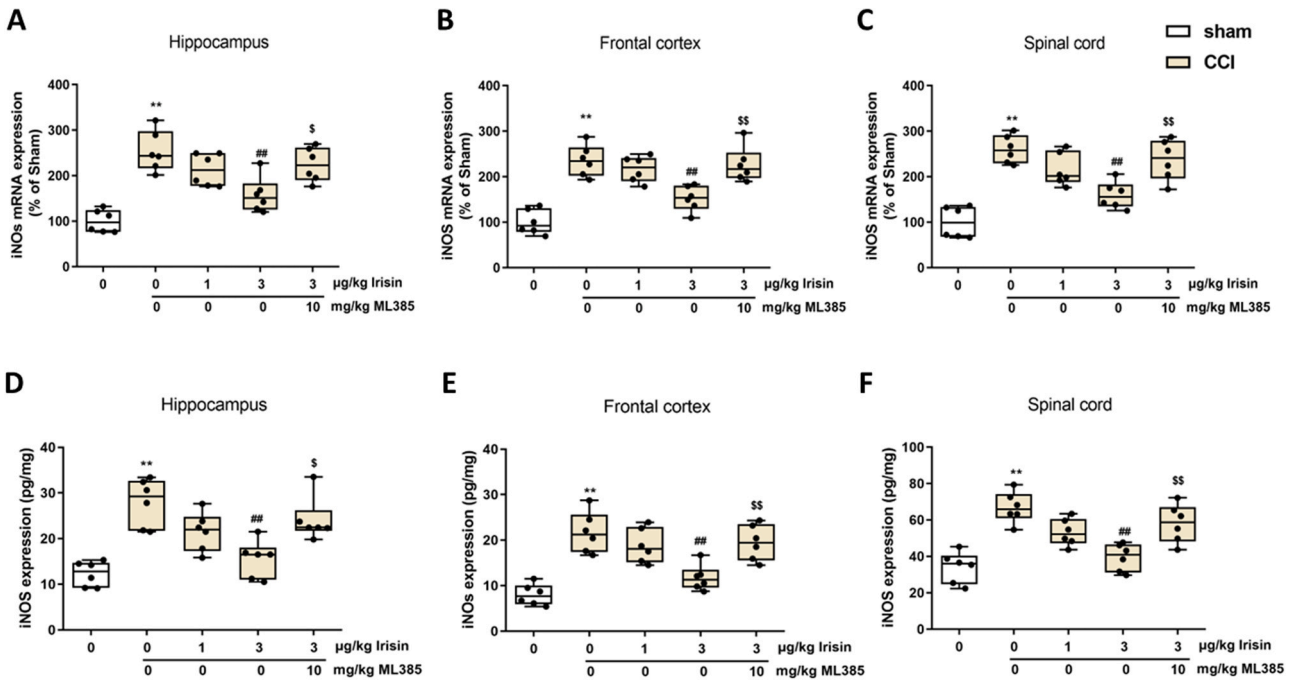

Fig. 7. Effects of irisin on iNOS mRNA(A-C) and protein (D-F) expressions in the hippocampus, frontal cortex, and spinal cord in CCI mice. n = 6 per group. $^{**}p < 0.01$ versus sham; $^{\#\#}p < 0.01$ versus CCI; $^{\$}p < 0.05$ and $^{\$\$}p < 0{\cdot}01$ versus irisin (3 μg/kg).

Table 2
Effect of irisin on IL-1β, IL-6 and TNF-α expressions in hippocampus.

Group	Irisin Dose (μg/kg)	Hippocampus (pg/mg per tissue)		
		IL-1β	IL-6	TNF-α
Sham		6.8±1.2	7.8±1.0	7.2±1.1
CCI		18.0 ±2.1**	17.8 ±1.6**	17.9 ±1.8**
Irisin+CCI	1	12.2±1.9	12.1±1.9	11.5±2.5
	3	7.4±1.5$^{\#\#}$	9.0±1.0$^{\#\#}$	7.6±1.1$^{\#\#}$
ML385+irisin+CCI		14.5±1.7$^{\$}$	15.6±1.0$^{\$}$	15.7±1.6$^{\$}$

Values are expressed as mean ± SEM for 8 mice in each group. Data analysis was performed using Dunnett's -test. ** $P < 0.01$ versus sham; $^{\#\#}$ $P < 0.01$ versus CCI; $^{\$}$ $P < 0.05$ versus irisin (3 μg/kg).

< 0.01 for IL-1β; $F(4,35)=9.84$, $p < 0.01$ for IL-6; $F(4,35)=7.87$, $p < 0.01$ for TNF-α; Table 2], frontal cortex [$F(4,35)=8.53$, $p < 0.01$ for IL-1β; $F(4,35)=7.22$, $p < 0.01$ for IL-6; $F(4,35)=9.99$, $p < 0.01$ for TNF-α; Table 3], and spinal cord[$F(4,35)=8.84$, $p < 0.01$ for IL-1β; $F(4,35)=7.58$, $p < 0.01$ for IL-6; $F(4,35)=8.29$, $p < 0.01$ for TNF-α; Table 4]. The increases in proinflammatory cytokine levels were suppressed by irisin treatment. The effects of exogenous irisin on iNOS and inflammatory factors were abolished by ML385.

Table 3
Effects of irisin on IL-1β, IL-6 and TNF-α expressions in frontal cortex.

Group	Irisin Dose (μg/kg)	Frontal cortex (pg/mg per tissue)		
		IL-1β	IL-6	TNF-α
Sham		7.2±1.1	6.1±0.8	7.0±1.0
CCI		15.2 ±1.7**	14.1 ±1.2**	16.0 ±1.4**
Irisin+CCI	1	9.4±1.1	10.2±1.9	10.1±1.5$^{\#}$
	3	7.8±0.9$^{\#\#}$	8.1±1.2$^{\#}$	8.5±1.1$^{\#\#}$
ML385+irisin+CCI		13.7±1.2$^{\$}$	13.6±1.0$^{\$}$	14.9±1.2$^{\$\$}$

Values are expressed as mean ± SEM for 8 mice in each group. Data analysis was performed using Dunnett's-test. ** $P < 0.01$ versus sham; $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$ versus CCI; $^{\$}$ $P < 0.05$ and $^{\$\$}$ $P < 0.01$versus irisin (3 μg/kg).

Table 4
Effects of irisin on IL-1β, IL-6 and TNF-α expressions of spinal cord.

Group	Irisin Dose (μg/kg)	Spinal cord (pg/mg per tissue)		
		IL-1β	IL-6	TNF-α
Sham		7.8±1.8	6.1±1.1	6.3±1.2
CCI		19.6 ±1.9**	14.5 ±1.6**	15.2 ±1.7**
Irisin+CCI	1	12.8±1.8	9.6±1.1	11.8±1.4
	3	8.7±1.6$^{\#\#}$	7.6±1.2$^{\#\#}$	8.7±0.9$^{\#}$
Ml385+irisin+CCI		17.9±1.8$^{\$\$}$	13.1±1.4$^{\$}$	15.7±1.7$^{\$}$

Values are expressed as mean ± SEM for 8 mice in each group. Data analysis was performed using Dunnett's -test. ** P < 0.01 versus sham; # P < 0.05 and ## P < 0.01 versus CCI; $ P < 0.05 versus irisin (3 μg/kg).

4. Discussion

In the present study, we used CCI mice model to explore whether irisin possess potential therapeutic effects on chronic neuralgia and the associated neurological disorders by performing a series of behavior tests. As surgical or medical treatment may suppress or stimulate the function of the central nervous system (CNS), leading to increased or decreased locomotor activity. The changes in animal locomotor activity may influence the results of behavioral tests (Singh et al., 2011). Hence, in our experiments, each mouse was subjected to the locomotor activity test before the behavioral tests to determine whether CCI or sham surgery and irisin treatment have impacts on the autonomous activities of mice. Results showed that there is no significant difference among the sham, CCI, and irisin-treated groups regarding the locomotor activity, indicating that neither CCI or sham surgery nor irisin treatment affected the measurement accuracy of the behavioral tests.

The CCI model is an established pain-related animal model that can simulate clinical symptoms of chronic neuralgia in humans (Barrot, 2012). The emotional deficit is a comorbid disease condition of CCI animals with hyperalgesia (X. Jiang et al., 2018). The results of thermal and mechanical hyperalgesia tests showed that CCI-treated mice exhibited hyperalgesia and the behavior tests showed that the CCI mice suffered from emotional deficits (depression and anxiety) as compared

with the sham mice, indicating that the CCI animal model was successfully constructed in this study.

In addition to hyperalgesia, depression, and anxiety, changes in CCI mice also include the decreases of endogenous irisin in serum, cerebrospinal fluid, and spinal cord, indicating that the low level of irisin in CCI mice may be a contributing factor for the chronic neuralgia. When we treated CCI mice with exogenous irisin, the hyperalgesia, depression, and anxiety were all attenuated, suggesting that irisin have therapeutic effects on chronic neuralgia and the associated neurological disorders. Previous studies have found that neuroinflammation is one of the pathological mechanisms of chronic neuralgia, and it is closely related to the onset of emotional disorders(Lees et al., 2015; X. G. Liu, 2022; Walker et al., 2014). As NF-κB plays a crucial role in the regulation of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α(Csaki et al., 2009), we tested the NF-κB, IL-1β, IL-6, and TNF-α levels in the hippocampus, frontal cortex, and spinal cord of mice in different groups to explore the possible anti-inflammation activity of irisin. Results showed that NF-κB was activated and the IL-1β, IL-6, and TNF-α levels were enhanced by CCI treatment. This was in consistent with the results in a previous study that TNF-α and IL-6 in the spinal cord of the animal could be enhanced by CCI treatment (Sakhaee et al., 2020). Moreover, the overexpression of NF-κB and IL-1β, IL-6, and TNF-α levels were accompanied by the abnormality of behaviors. The changes in levels of inflammatory cytokines and the behaviors were reversed by chronic irisin treatment. These results suggest neuroinflammation is involved in the development of CCI-induced chronic neuralgia and the associated neurological disorders and irisin's therapeutic effects on CCI mice was associated with its anti-inflammatory function.

NF-κB is not only the regulator of proinflammatory cytokines, but also the regulator of neurotoxic substances such as NOS and COX-2 (Csaki et al., 2009). NOS is an enzyme expressed in three isoforms (nNOS, iNOS, and eNOS). These isoforms are reported to be expressed in the CNS (Wegener and Volke, 2010). Nitric oxide derived from iNOS and nNOS shows neurotoxic effects, while nitric oxide derived from eNOS has neuroprotective effects (Anaeigoudari et al., 2016). In addition, a previous study suggested that specific genetic variants of these NOS isoforms may increase the risk of affective disorders and neuralgia (Cinelli et al., 2020; Zhou et al., 2018). Based on the previous findings, we explored whether neurological iNOS is also involved in the pathogenesis of chronic pain and emotional deficits in CCI mice. According to the results, iNOS levels were remarkably higher in the hippocampus, frontal cortex, and spinal cord of the CCI mice, while irisin ameliorated the overexpression. This phenomenon suggests that iNOS is associated with affective disorders and neuralgia and the irisin's positive effects on depression, anxiety, and hyperalgesia might be related to the inhibition iNOS through the NF-κB signaling pathway.

In addition to inflammation and iNOS, oxidative stress is also one of the pathological mechanisms of chronic neuralgia and the relevant emotional disorders. It was reported that chronic neuralgia can stimulate the generation of reactive oxygen species, resulting in the occurrence of central oxidative stress; this process acts as one of the pathogenetic mechanisms of the development of CCI-induced behavioral abnormalities such as depression and anxiety(Xu et al., 2014). Oxidative stress mediators such as MDA, SOD, and GSH are reported to be involved in the progression of oxidative stress triggered by CCI (Kaushik et al., 2020). In one hand, the occurrence of chronic neuralgia resulted in an elevation in the level of MDA, the final product of lipid peroxidation. This elevation can serve as an indirect indicator of the magnitude of damage caused by free radicals (Sakhaee et al., 2020). In the other hand, chronic neuralgia hinders the capabilities of GSH and SOD in scavenging lipid peroxides and oxygen free radicals (Wang et al., 2019). Thus, in our study, levels of MDA, GSH, and SOD in the hippocampus, frontal cortex, and spinal cord of the CCI mice were tested. The results showed that MDA level significantly increased and GSH and SOD levels significantly decreased, which was consistent with the results of a previous study that CCI treatment could enhance MDA level and suppress GSH level in the spinal cord of CCI animals (Amin et al., 2014). The changes of MDA, GSH, and SOD levels are accompanied by the changes in the behavior tests, which confirmed the involvement of oxidative stress in the pathological mechanism of chronic neuralgia and emotional disorders as reported previously. Irisin reversed the abnormal expressions of MDA, GSH, and SOD, suggest that irisin's therapeutic effects was associated with its anti-oxidative stress function.

Nrf2 is a transcription factor responsible for regulating the expression of genes that encode antioxidant enzymes and proteins (Tonelli et al., 2018). In the normal state, the activity of Nrf2 is suppressed through binding to Kelch-like ECH-associated protein 1 (KEAP1). In the presence of oxidative stress, Nrf2 separates from KEAP1, translocates to the nucleus, and binds to the antioxidant response element; this process promotes the transcription of numerous antioxidant genes, such as SOD (Bellezza et al., 2018). In order to explore whether Nrf2 is involved in the CCI-relevant pathology, Nrf2 levels in the hippocampus, frontal cortex, and spinal cord of the CCI mice were measured. Results showed that the expression of Nrf2 in CCI mice significantly decreased, which was in consistent with Sun et al.'s finding that CCI decreases the nuclear levels of Nrf2 (Sun et al., 2021). The decline of Nrf2 was accompanied by the abnormality of behaviors. It is reasonably to speculate that CCI induced the decrease of Nrf2 first and then the antioxidant genes such as SOD were suppressed, leading to the progression of oxidative stress and the neurological disorders. Our results showed that the decline of Nrf2 was effectively counteracted by the administration of irisin; moreover, the ameliorative effects of irisin on CCI-induced chronic neuralgia and the comorbid affective disorders were reversed by the Nrf2 antagonist ML385. Collectively, the therapeutic effects of irisin on CCI-relevant pathology is associated with Nrf2 and the ability of irisin to mitigate oxidative stress may be reliant on the activation of the Nrf2 signaling pathway.

5. Conclusion

The present study showed that irisin can ameliorate hyperalgesia and emotional deficits in CCI mice. The neuroprotective properties of irisin may be attributed to its ability to enhance the oxidative stress response via the Nrf2-mediated antioxidative stress pathway, as well as its notable ability to suppress inflammatory mediators and iNOS by modulating the NF-κB signaling pathway (Fig. 8). Because of these functions, irisin could be considered as a potential therapeutic target for CCI-induced nociceptive hyperalgesia and the associate affective disorders.

Compliance with ethical standards

All the experimental protocols were approved by the Animal Care and Use Committee of Zhejiang Pharmaceutical University and conducted in accordance with the regulations set by the Committee.

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CRediT authorship contribution statement

Hanqin Zhang: Writing – original draft. **Xuefeng Yu:** Investigation. **Xupei Xie:** Investigation. **Fan Wu:** Conceptualization. **Yuyang Huang:** Investigation. **Huidan Dai:** Writing – review & editing.

Declaration of Competing Interest

All the authors declare that they have no conflict of interest.

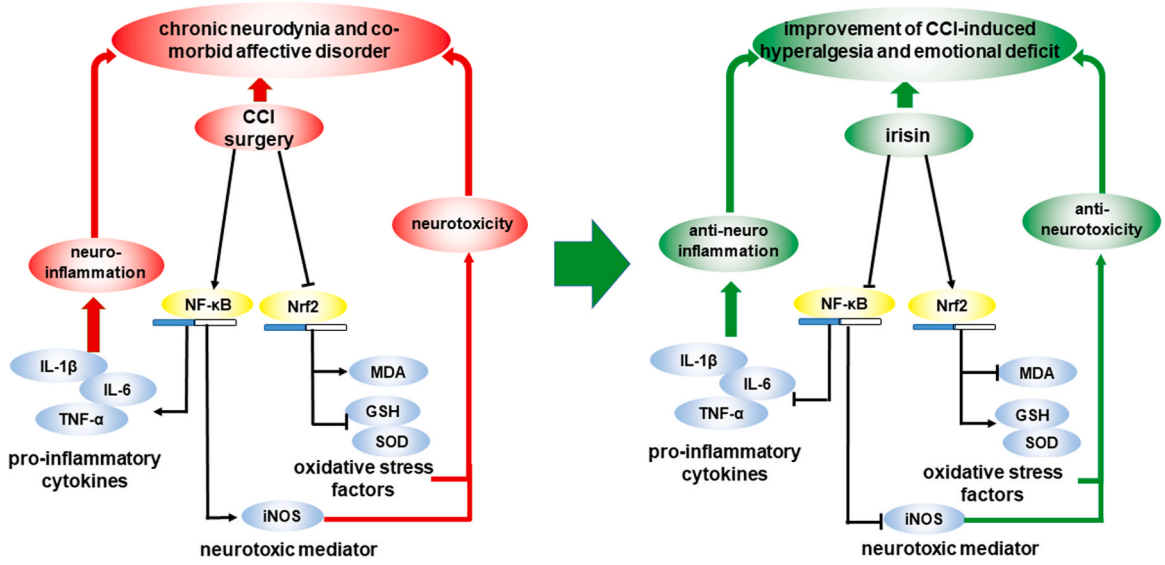

Fig. 8. Molecular mechanisms underlying the protective effects of irisin against neuroinflammation and neurotoxicity induced by CCI. The effects of irisin on depressive and anxiety-like behaviors induced by CCI may be attributed to its ability to inhibit NF-κB and proinflammatory cytokines. The other potential mechanism underlying the neuroprotective effects of irisin against depressive and anxiety-like behaviors induced by CCI may involve the activation of Nrf2 and the amelioration of oxidative stress, as evidenced by a reduction in the MDA level and increases in SOD and GSH levels.

Data Availability

The data of this study can be obtained from the corresponding author upon reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibneur.2024.08.009.

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