# High-Intensity Interval Training Increases Osteoarthritis-Associated Pain-Sensitive Threshold Through Reduction of Perineuronal Nets of the Medial Prefrontal Cortex in Rats

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**Summary**

High-intensity interval training (HIIT) is considered an effective therapy strategy for improving chronic pain associated with osteoarthritis (OA). Perineuronal nets (PNNs) are specialized extracellular matrix structures in the cerebral cortex that play a crucial role in regulating chronic pain. However, little is unknown whether HIIT could alleviate OA pain sensitization by reducing PNN levels. This study aimed to determine whether HIIT could reduce sensitivity of the affected joint(s) to pain in a chronic pain model in rats with OA. A rat model of interest was induced by intra-articular injection of monosodium iodoacetate (MIA) into the right knee. Thereafter, the mechanical withdrawal thresholds (MWTs) and PNN levels in the contralateral medial prefrontal cortex (mPFC) were measured in rats in the presence or absence of HIIT alone or in combination with injection of chondroitinase-ABC (ChABC) into the contralateral mPFC (inducing the degradation of PNNs), respectively. Results indicated that rats with OA exhibited significant reductions in MWTs, but a significant increase in the PNN levels; that HIIT reversed changes in MWTs and PNN levels in rats with OA, and that pretreatment of ChABC abolished effects of HIIT on MWTs, with PNN levels not changed. We concluded that pain sensitization in rats with OA may correlate with an increase in PNN levels in the mPFC, and that HIIT may increases OA pain-sensitive threshold by reduction of the PNN levels in the mPFC.

**Keywords**

Osteoarthritis • Chronic pain • Pain sensitization • High-intensity interval training • Perineuronal nets

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## Introduction

Chronic pain is one of the main symptoms of osteoarthritis (OA) and impairs patients' quality of life to a greater extent [1]. However, the etiology of chronic pain in OA remains unclear. Pain sensitization is considered a key process in chronic pain and is characterized by an exaggerated response to harmless or only mildly harmful stimuli [2]. Pain sensitization in OA includes peripheral sensitization, which results from the hyperexcitability of peripheral nociceptors [3,4], and central sensitization, which arises from the enhanced reactivity of nociceptive neurons within the central nervous system to normal or subthreshold stimuli due to neuroplasticity [5]. Studies have shown that pain sensitization leads to chronicity and treatment resistance in OA pain [6-10]. However, the molecular targets involved in OA-associated pain sensitization are poorly understood. Identifying effective therapeutic targets is crucial for alleviating chronic pain associated with OA.

Perineuronal nets (PNNs) are specialized extracellular matrix structures primarily composed of chondroitin sulfate proteoglycans (CSPGs), such as aggrecan, brevican, neurocan, hyaluronic acid, tenascin-R, and other linked proteins [11]. In the cerebral cortex, PNNs predominantly surround $PV^+$ inhibitory interneurons and regulate neural plasticity under physiological or pathological conditions [12]. During specific



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physiological conditions, the developmental maturation of PNNs coincides with the closure of critical periods of neural plasticity in certain brain regions. The degradation of PNNs mediated by chondroitinase treatment can reopen these critical periods [13], highlighting the involvement of PNNs in modulating neural and developmental plasticity. Similarly, PNNs play a crucial role in regulating certain pathological processes associated with neural plasticity [12]. An increase in PNN density around insular neurons in mice with chronic alcoholism can restrict neuronal plasticity and contribute to compulsive drinking behavior [14]. Furthermore, combining withdrawal training with targeted degradation of PNNs within the amygdala can effectively erase drug memories related to heroin and cocaine use and reduce relapse behaviors [15]. It is well known that pain sensitization is a form of pathological neural plasticity. Although studies on PNNs and pain sensitization are scarce, relevant evidence has shown that PNNs may be closely related to pain sensitization [16]. Disruption of PNNs can reduce the excitability of inhibitory interneurons and increase the release of painful neurotransmitters, thereby causing pain responses or sensitization [17,18]. Similarly, abnormal PNN levels may be related to chronic pain in animal models of traumatic brain injury [19]. Therefore, exploring the role of PNNs in OA-associated pain sensitization would provide new therapeutic targets for the management of chronic pain associated with OA.

High-intensity interval training (HIIT) is an exercise method characterized by alternating short periods of intense activity with rest or active recovery. Previous studies have demonstrated the effectiveness of HIIT in improving pain and clinical symptoms in individuals with OA [20,21]. However, little is known about the underlying mechanisms by which HIIT would alleviate OA pain sensitization. The medial prefrontal cortex (mPFC), which is a crucial cortical area involved in pain regulation, has been shown to play a significant role in chronic pain in OA patients based on functional MRI studies [22,23]. Recent basic research has also revealed that abnormal levels of PNNs in the mPFC are associated with chronic inflammatory pain induced by the injection of complete Freund's adjuvant (CFA) in the unilateral hind paws of mice [24]. Although there have been reports suggesting that exercise training can modulate PNN levels and improve motor function in rats with spinal cord injury [25-27], it remains unclear whether HIIT can improve pain sensitization in rats with OA by regulating PNNs levels within the mPFC. Therefore, this study aimed to elucidate the mechanisms by which HIIT could regulate changes in PNN levels within the mPFC to reduce pain sensitization in a chronic pain rat model after induction of OA of the right knee joint by local injection of monosodium iodoacetate (MIA).

## Materials and Methods

All animal care and experimental procedures were approved by the Experimental Animal Welfare and Ethics Committee, Nanjing First Hospital, China, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals, released by the U.S. National Institutes of Health.

*OA model*

Male Sprague-Dawley rats (aged 8 weeks old and weighing 200 ± 20 g) were housed in the ventilated cages (46 × 35 ×20 cm each). Rats were maintained in an air-conditioned room (maintaining at 24 ± 2 °C and a humidity of 50 ± 5 %), with a light/dark cycle alternated every 12 h, Each cage accommodated 4 rats. Food and tap water were provided ad libitum. After acclimatization for 1 week, rats were anesthetized by an intraperitoneal (i.p.) injection of 1.5 % pentobarbital sodium (30 mg/kg). A single intra-articular injection of MIA (1 mg/50 μl sterile normal saline solution; Sigma-Aldrich, St. Louis, MO, USA) was given to the right knee joint as described elsewhere [28]. Control Rats received an equal volume of sterile normal solution saline (vehicle control) by intra-articular injection.

*Study design*

The experiments included three phases. In the first phase, a total of 24 rats were randomly allocated into two groups: the control group and the OA group (n = 12 each). The OA group was administered an intra-articular injection of 50 μl of MIA (1 mg dissolved in 50 μl of normal saline solution) in the right knee joint to establish the OA model as described elsewhere [28], while the control group was injected with an equal volume of normal saline solution as a vehicle control. In the second phase, 24 rats were randomly divided into two groups: the OA group and the OA + HIIT group (n = 12 each). The OA + HIIT group underwent a HIIT exercise regimen three times per week for four consecutive weeks, and each session lasted for 20 min. The OA group was placed on a stationary treadmill and allowed unrestricted movement which served as the control. In the third phase, a total of 60 rats were randomly assigned to five groups (n = 12 each): the

OA group, OA + chondroitinase-ABC (ChABC) group, OA + Vehicle group, OA + ChABC + HIIT group, and OA + Vehicle + HIIT group. Beginning at week four after OA induction, the OA + ChABC + HIIT and OA + Vehicle + HIIT groups underwent a 4-week HIIT regimen. Rats in the OA, OA + ChABC, and OA + Vehicle groups were placed on a stationary treadmill and allowed for free movement. One day before the first HIIT session each week, rats in the OA + ChABC and OA + ChABC + HIIT groups were injected with 0.6 μL of 100 U/mL ChABC by stereotaxic brain injection to digest PNNs in the contralateral mPFC, while the OA + Vehicle group and OA + Vehicle + HIIT group received an equal volume of ChABC vehicle (PBS containing 0.1 % BSA) as a control. Injections of ChABC or vehicle were administered once per week for a total of 4 weeks. Mechanical pain threshold testing was conducted the following designated time points: at the 4$^{th}$ week after modeling and after four weeks of HIIT, respectively. After measurement of the final pain threshold, rats were euthanized for subsequent tissue acquisition and molecular biology studies.

*HIIT Protocol*

The HIIT protocol was based on a protocol that was previously reported [29]. Prior to commencing the official HIIT protocol, rats underwent a 7-day acclimatization period on the treadmill. During this period, the exercise speed and intensity were gradually increased until all the rats became accustomed to the treadmill environment and the adjustments in speed. Rats that failed to adapt to treadmill running during the familiarization process were promptly excluded and replaced, ensuring completion of the HIIT protocol in each group of rats. The HIIT protocol included a 5-min warm-up phase with an incremental speed that increased from 5 m/min to 15 m/min, followed by high-intensity and moderate-intensity exercise intervals, with each alternated every 2 min, for a total of 6 cycles. Each cycle consisted of 30-sec high-intensity exercise at a speed of 26 m/min, followed by 90-sec moderate-intensity exercise at 15 m/min, resulting in a total duration of 2 min each cycle. The session concluded with a 3-min cool-down phase at a speed of 5 m/min. Each session lasted for a total of 20 min and was conducted 3 times weekly for 4 weeks.

*Stereotaxic injection of ChABC in the brain*

After the rats were anesthetized by an i.p. injection, they were fixed onto a stereotaxic injection device. The scalp was cut to expose the skull, and a 3 % H2O2 (hydrogen peroxide) solution was used to clean the tissue on the skull, ensuring full exposure of the anterior fontanelle, which served as the reference point for the injection. The injection site was determined based on the coordinates where ChABC was to be injected as follows: A) AP: + 3.0 mm; ML: 0.6 mm; DV: -4.2 mm; B) AP: +3.0 mm; ML: 0.6 mm; DV: -4.0 mm; and C) AP: +3.0 mm; ML: 0.6 mm; DV: -3.8 mm). The tip of the microinjection needle was then moved to the corresponding sites and marked, respectively. A small hole was then drilled through the skull at the marked sites using an electric drill, and the drilling debris was promptly removed. The microinjection needle was inserted into the brain at the designated coordinates, and slow injection of ChABC (100 U/ml) of 0.6 μl or the vehicle (PBS containing 0.1 % BSA) was performed, respectively. After that, the needle was left in situ for 5 min prior to gradual withdrawal. The cranial defect was meticulously filled with bone wax, followed by meticulous suturing of the scalp. Subsequently, after recovering from anesthesia, the rats were reintroduced into their corresponding cages. The injections were administered one day before a HIIT session weekly, for a total of 4 injections [30].

*Mechanical pain threshold assessment*

The mechanical withdrawal threshold (MWT) of rats was assessed using the von Frey test (NC12775-99, North Coast, USA). The rats were placed in a custom-made organic glass compartment with a grid floor and allowed to acclimate for approximately 20 min until their limb movements were reduced. A series of von Frey filaments (0.4, 0.6, 1.4, 2, 4, 6, 8, and 15 g) were sequentially used to stimulate the skin on the middle part of the right hind paw using the 'Up & Down' method. Each filament was bent into either a 'C' or 'S' shape and maintained for a duration of 6 to 8 sec. A lack of response was recorded as a negative response ('O'), while any foot withdrawal or licking behavior was considered a positive response ('X'). The mechanical pain threshold was calculated using the following formula: 50 % withdrawal threshold (g) = $[10^{(Xf+K\delta)}]/10{,}000$, where Xf represents the number corresponding to the last tested filament and K is the coefficient obtained from the table based on the 'X,' 'O' sequence, and $\delta = 0.224$ [31].

*Western blot analysis*

The mPFC brain tissue was lysed using RIPA lysis buffer (Beyotime, Shanghai, China), and the protein concentration was determined using a BCA protein assay

kit (Keygenbio, Nanjing, China). Subsequently, the protein samples were separated on a 4 % to 20 % SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with fast blocking solution (G2052, Servicebio, Wuhan, China) at room temperature for 5 min, followed by overnight incubation at 4 °C with the following primary antibodies: anti-ACAN (1:1000; 13880-1-AP, aggrecan polyclonal antibody, Proteintech, Wuhan, China), anti-BCAN (1:1000; ab285162, anti-brevican antibody, Abcam, Cambridge, UK), anti-NCAN (1:1000; ab277525, anti-neurocan antibody, Abcam, Cambridge, UK), anti-SP (1:1000; DF7522, substance P antibody, Affinity Biosciences, Jiangsu, China), anti-IL-1β (1:1000; bs-0812R, rabbit anti-IL-1β, Bioss, Beijing, China), or anti-β-actin (1:1000, GB15003, Servicebio, Wuhan, China). Finally, the membranes were incubated for 1.5 h with goat anti-rabbit IgG secondary antibodies (1:50,000; Biosharp, Guangzhou, China). The bands were visualized using an enhanced chemiluminescence reagent (UElandy, Suzhou, China) and quantified using ImageJ software [32].

*Immunofluorescence analysis*

Rat brain tissue was extracted and fixed with 4 % paraformaldehyde for 6 h. Subsequently, the tissue was dehydrated and paraffin embedded. Tissue sections of a 3-μm thickness were prepared. The sections were immersed in xylene I for 15 min, followed by xylene II for 10 min. Then, the sections were subjected to gradient dehydration in ethanol (100 %, 95 %, 80 %, or 75 %) for 1 min each and rinsed with distilled water for 3 min. Next, the tissue sections were exposed to antigen retrieval solution (pH 6.0; Servicebio, Wuhan, China) at 95 °C for 15 min and then allowed for return to room temperature. After being washed three times with PBS for 5 min each, the sections were blocked and permeabilized in a mixture of 3 % BSA serum containing 0.3 % Triton X-100 at room temperature for 1.5 h. The primary antibodies used included anti-PV (parvalbumin polyclonal antibody; Proteintech, Wuhan, China) and Wisteria floribunda agglutinin (WFA) (L1516; Sigma-Aldrich, St. Louis, MO, USA), which were added and incubated overnight at 4 °C. Subsequently, Cy3-labeled goat anti-rabbit secondary antibodies (1:200; Servicebio, Wuhan, China) and Alexa Fluor 488-conjugated streptavidin (1:200; Yeasen, Shanghai, China) were added and incubated for 1.5 h. The nuclei were stained using DAPI (G1012, Servicebio, Wuhan). Finally, the coverslips were applied using an anti-fluorescence quenching agent (G1401; Servicebio, Wuhan). Images were obtained by scanning microscopy (Carl Zeiss Microscopy GmbH, Germany) [33]. ImageJ software was used to quantitatively analyze the fluorescence intensity of PNNs and the number of PV+ interneurons.

*Immunohistochemistry analysis*

Right knee joint tissue was extracted and fixed with 4 % paraformaldehyde for 24 h. Subsequently, the tissue was decalcified, wax immersed, and paraffin embedded. Tissue sections of a 3-μm thickness were prepared. The sections were immersed in xylene I for 20 min, followed by xylene II for 20 min. Then, the sections were subjected to gradient dehydration in ethanol (100 %, 95 %, 80 %, or 75 %) for 1 min each and rinsed with distilled water for 3 min. Next, the tissue sections were exposed to antigen retrieval solution (pH 6.0; Servicebio, Wuhan, China) at 60 °C overnight and allowed to cool to room temperature. Then follow the steps described in the Elabscience two-step immunohistochemical kit (2-step plus poly-HRP anti-mouse/-rabbit IgG detection system, E-IR-R217, Elabscience, China) : dropping 3 % H2O2 for 10 min at room temperature to inactivate endogenous enzymes, and washed with PBS. Subsequently, they were incubated with normal goat blocking buffer and sealed at 37 °C for 30 min, with excess liquid removed by shaking. Dropping primary antibodies: anti-COL2A1 (1: 200; sc-52658, Santa Cruz, Texas, USA), anti-MMP13 (1: 200; 18165-1-AP, MMP13 polyclonal antibody, Proteintech, Wuhan, China), incubating at 37 °C for 1.5 h, washed with PBS, dropping polyperoxidase-anti-mouse/rabbit IgG and incubating at 37 °C for 30 min, washed with PBS. DAB staining showed a brownish yellow color, and the sections were washed with distilled water to stop staining. The sections were stained with hematoxylin for 5 min, then stained with hematoxylin differentiation solution for 5 sec, and hematoxylin blue return solution for 5 sec. Finally, the samples were dehydrated with ethanol of 75 %, 80 %, 95 %, or 100 % for 3 min each, followed by xylene I and II for 5 min each, and then sealed. Images were obtained by scanning microscopy (OLYMPUS BX51 microscope, Japan) [34]. Quantitative analysis was performed using ImageJ software (NIH, MD, USA).

*Statistical analysis*

The normality of the data was assessed using the Shapiro-Wilk test. The data are presented as the mean ± SD and were analyzed using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA). Comparisons between two groups were performed using

unpaired Student's t test. Comparisons among more than two groups were performed using one-way ANOVA, followed by post hoc Tukey test . A P value of less than 0.05 was considered statistically significant.

## Results

*PNNs and inflammatory pain substances levels in the mPFC of rats with OA*

In the 4th week after modeling, the mechanical pain threshold (Fig. 1A) on the operated side was significantly lower in the OA group than in the control group (*P<0.001*). Immunofluorescence staining was also performed to analyze PNN levels in the contralateral mPFC, including the anterior cingulate, prelimbic, and infralimbic cortex regions (Fig. 1B-C). The density of $WFA^+$ PNNs and the number of $WFA^+/PV^+$ cells were significantly increased in the contralateral mPFC of rats with OA compared to those in the control group (*P<0.001*). Additionally, Western blot analysis was performed to evaluate the protein expression levels of key PNN components (ACAN, BCAN, and NCAN), as well as inflammatory pain substances (SP and IL-1β), in the contralateral mPFC region (Fig. 1D). The protein expression levels of BCAN and NCAN, SP and IL-1β were significantly increased in the contralateral mPFC of rats with OA compared to those in the control rats (*P<0.001*). However, there were no significant changes in ACAN protein expression levels (*P>0.05*).

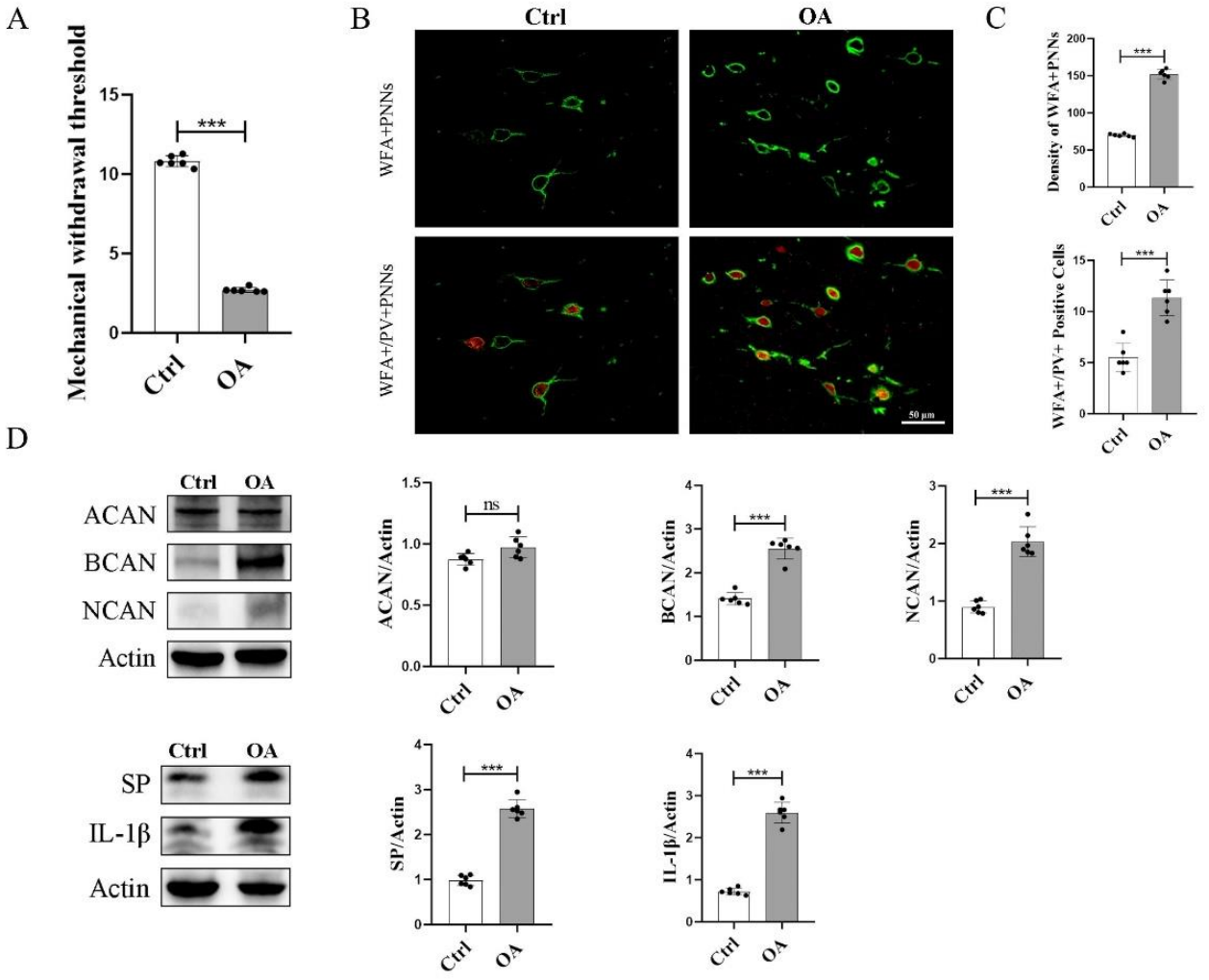


**Fig. 1.** Levels of PNNs and inflammatory pain substances in the mPFC of rats with OA compared with control rats (n = 6 each).
**A**: Changes in the mechanical pain threshold of rats. **B** and **C**: Representative immunofluorescence images (scale bar = 50 μm) and quantitative analysis of $WFA^+$ PNNs and $WFA^+/PV^+$ cells in the contralateral mPFC, including the anterior cingulate, prelimbic, and infralimbic cortex of control and OA rats. Green indicates $WFA^+$ PNNs, while red represents $PV^+$ interneurons. **D**: Representative Western blot images and quantitative analysis of the protein expression of the main components of PNNs (ACAN, BCAN, and NCAN) and inflammatory pain substances (SP and IL-1β) in the contralateral mPFC in control and OA rats. *** *P < 0.001*; ns, denotes no significant difference.

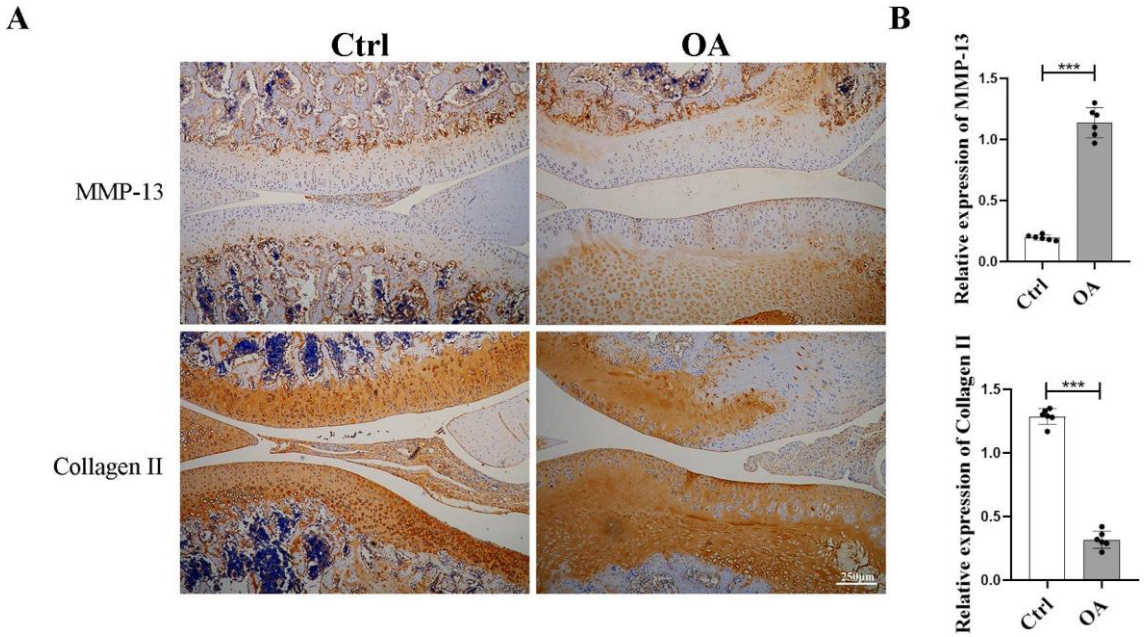


**Fig. 2.** MMP-13 and collagen II levels in the cartilage of rats with OA. **A** and **B** (n = 6 each): Representative images and quantification of MMP-13 and collagen II in cartilage by Immunohistochemistry staining. Scale bar = 250 μm. **** P<0.001*.

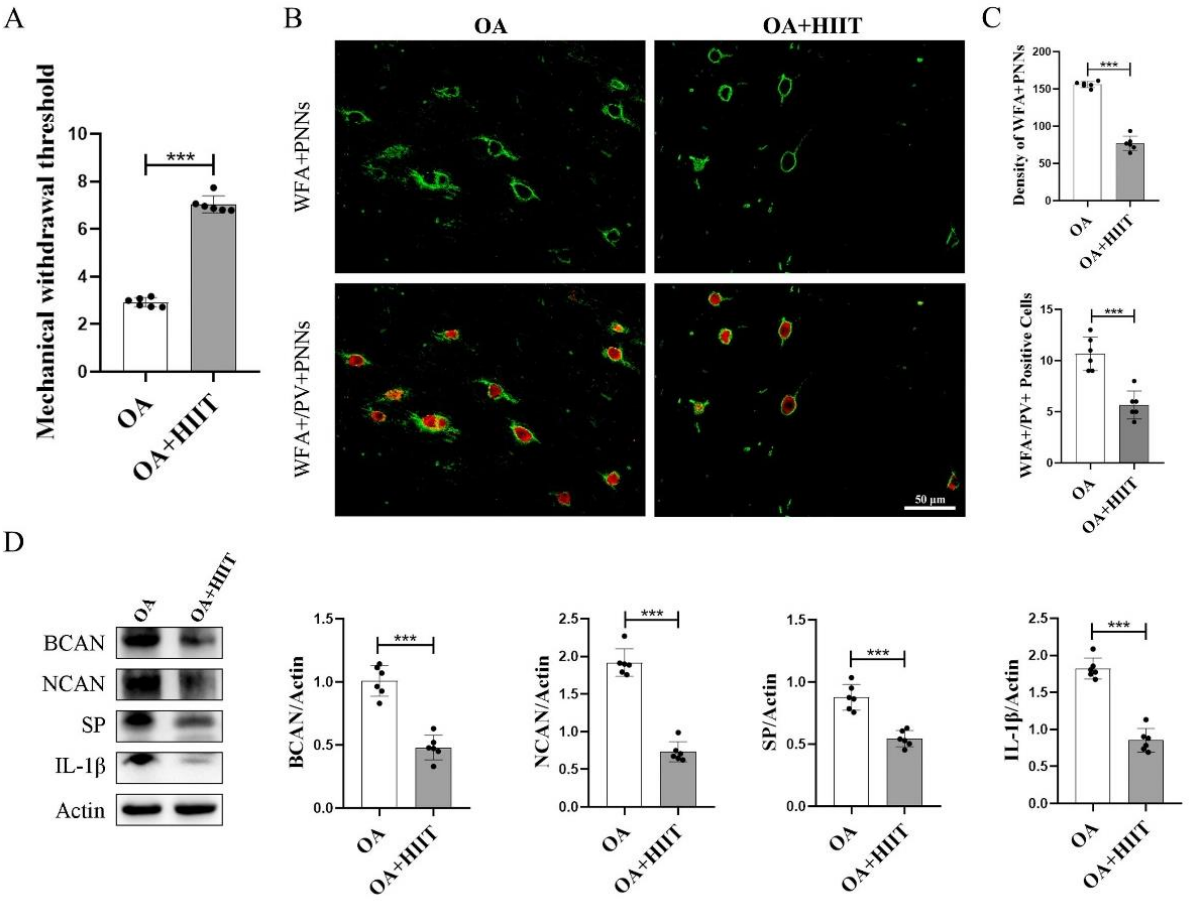


**Fig. 3.** The impact of HIIT on PNNs and inflammatory pain substances in the mPFC of rats (n = 6 each). **A**: Changes in the mechanical pain threshold of rats. **B** and **C**: Representative immunofluorescence images (scale bar = 50 μm) and quantitative analysis of $WFA^+$ PNNs and $WFA^+/PV^+$ cells in the contralateral mPFC, including the anterior cingulate, prelimbic, and infralimbic cortex, of OA alone vs. OA + HIIT groups. Green indicates $WFA^+$ PNNs, while red represents $PV^+$ interneurons. **D**: Representative Western blot images and quantitative analysis of the protein expression of the main components of PNNs (BCAN and NCAN) and inflammatory pain substances (SP and IL-1β) in the contralateral mPFC of OA alone and OA + HIIT groups. **** P<0.001*.

*MMP-13 and collagen II levels in the cartilage of rats with OA*

In the 4th week after modeling, the Immunohistochemistry staining was performed to analyze MMP-13 and collagen II levels in the cartilage of rats with OA (Fig. 2). Compared to the control group, the OA group exhibited reduced expression of collagen II but increased expression of MMP-13.

*The impact of HIIT on PNNs and inflammatory pain substances in the mPFC*

After 4 weeks of HIIT, the mechanical pain threshold (Fig. 3A) on the operated side was significantly higher in the OA + HIIT group than in the OA group (*P<0.001*). Immunofluorescence staining was used to analyze PNN levels in the contralateral mPFC, including the anterior cingulate, prelimbic, and infralimbic cortices (Fig. 3B-C). The density of $WFA^+$ PNNs in the contralateral mPFC of rats in the OA + HIIT group was significantly reduced compared to that in the OA group (*P<0.001*), and there was a notable decrease in $WFA^+$/$PV^+$ cell counts (*P<0.001*). Furthermore, Western blot analysis was performed to evaluate the protein expression levels of key components involved in PNN formation (BCAN and NCAN) and inflammatory pain substances (SP and IL-1β) in the contralateral mPFC region (Fig. 3D). Significantly reduced protein expression levels of BCAN, NCAN, SP, and IL-1β were measured within this region in rats in the OA + HIIT group compared to those in the OA group (*P < 0.001*).

*The impact of HIIT on MMP-13 and collagen II in the cartilage of rats with OA*

After 4 weeks of HIIT, the Immunohistochemistry staining was used to analyze MMP-13 and collagen II levels in the cartilage of rats with OA. Compared to the OA group, the OA + HIIT group exhibited reduced expression of MMP-13 but increased expression of collagen II. (Fig. 4).

*The effect of ChABC injection combined with HIIT on the levels of PNNs and inflammatory pain substances and pain sensitization in the mPFC of rats with OA*

The HIIT intervention was conducted on rats following digestion of PNNs in the contralateral mPFC using ChABC. In comparison to the OA group, the mechanical pain threshold of rats in the OA + ChABC group exhibited a significant increase (*P<0.001*) (Fig. 5A). Immunofluorescence staining was also performed to analyze PNN levels in the contralateral mPFC (Fig. 5B-C). The density of $WFA^+$ PNNs and the number of $WFA^+/PV^+$ cells were significantly decreased in the contralateral mPFC of rats in OA + ChABC group compared to those in OA group (*P<0.001*). Additionally, Western blot analysis was performed to evaluate the protein expression levels of key PNN components (BCAN,

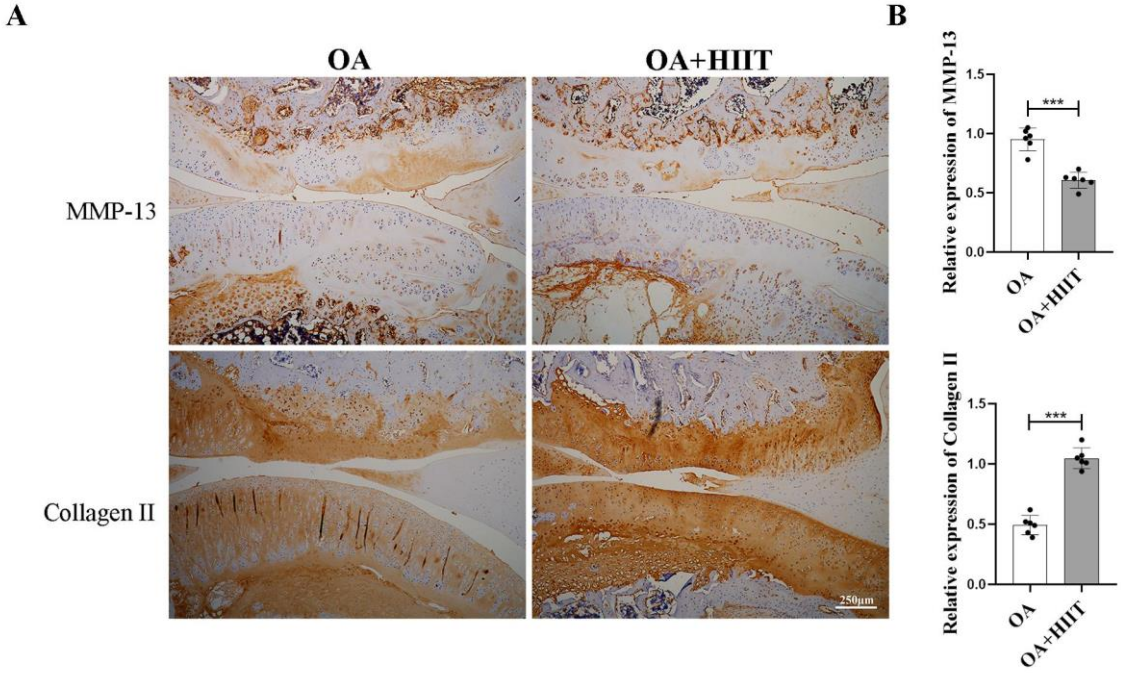


**Fig. 4.** The impact of HIIT on MMP-13 and collagen II in the cartilage of rats with OA. **A** and **B** (n = 6 each): Representative images of MMP13 and collagen II in cartilage by Immunohistochemistry staining. Scale bar = 250 μm. **** P<0.001.*

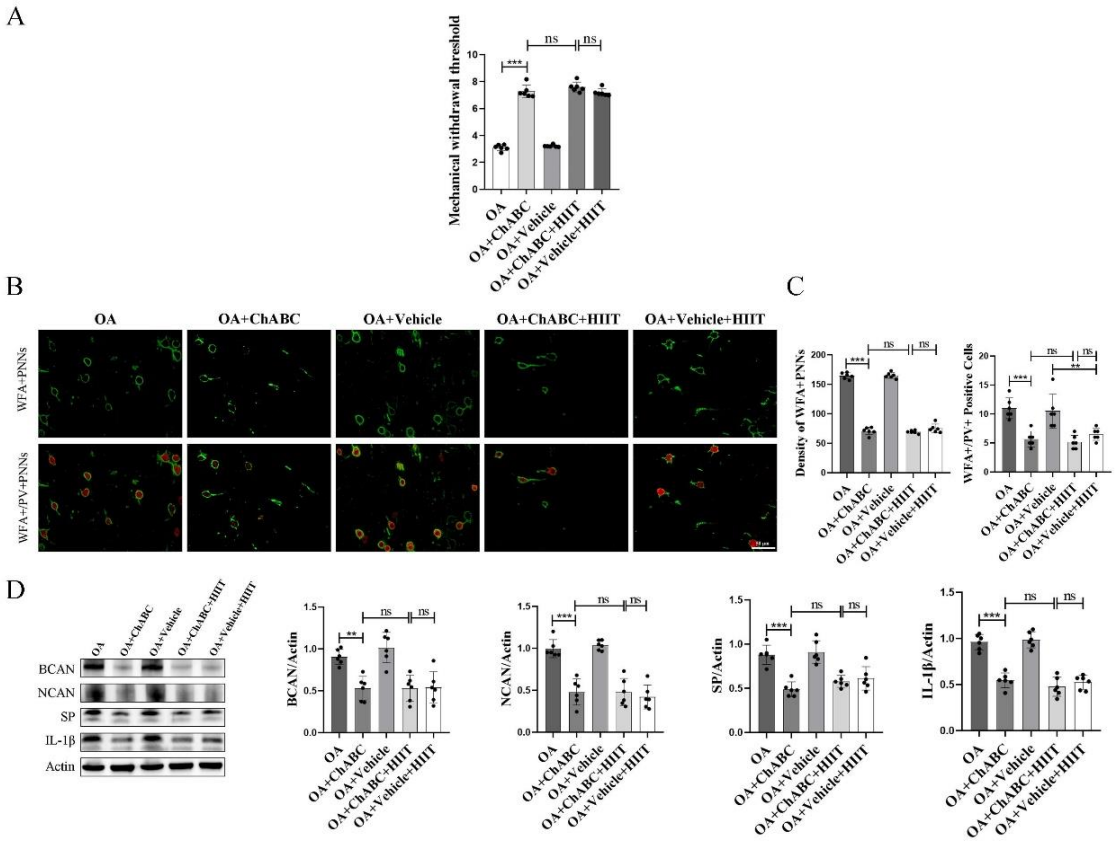


**Fig. 5.** The effect of ChABC injection combined with HIIT on the levels of PNNs and inflammatory pain substances and pain sensitization in the mPFC of rats with OA. **A**: Changes in the mechanical pain threshold of rats. **B** and **C**: Representative immunofluorescence images (scale bar = 50 μm) and quantitative analysis of $WFA^+$ PNNs and $WFA^+/PV^+$ cells in the contralateral mPFC, including the anterior cingulate, prelimbic, and infralimbic cortex, in each group. Green indicates $WFA^+$ PNNs, while red represents $PV^+$ interneurons. **D**: Representative Western blot images and quantitative analysis of the protein expression of the main components of PNNs (BCAN and NCAN) and inflammatory pain substances (SP and IL-1β) in the contralateral mPFC in each group. *** P < 0.01; *** P < 0.001*; ns denotes no significant difference.

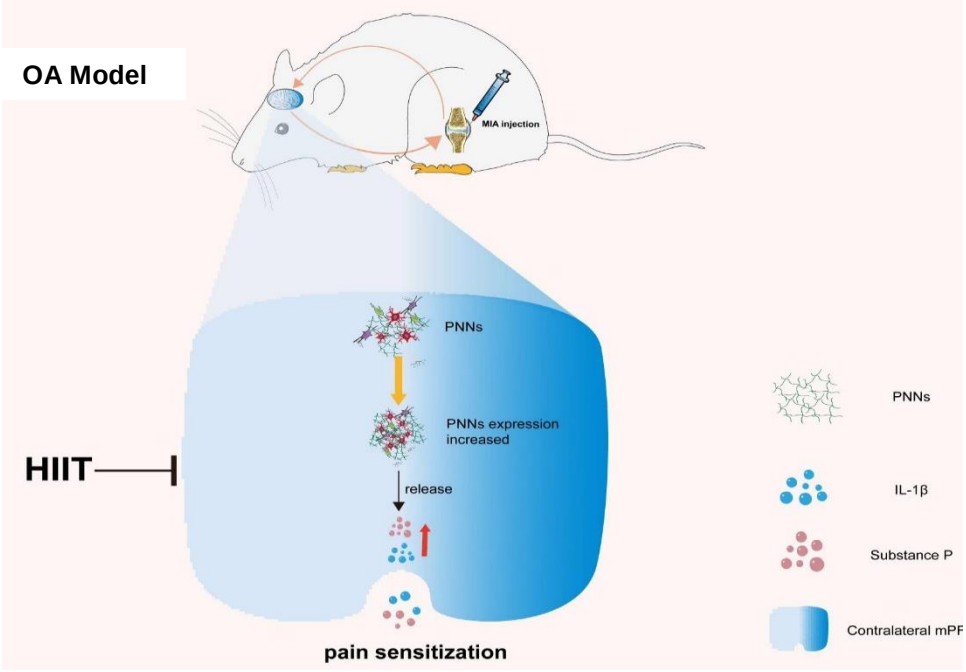


**Fig. 6.** Schematic illustration shows the mechanism through which HIIT modulates PNNs in the mPFC to ameliorate pain sensitization in OA. Inflammatory stimulation of the knee joint enhances peripheral nociceptor excitability, leading to continued transmission of pain signals via afferent nerves to the mPFC, thereby inducing central sensitization. It is characterized by increased expression of PNNs and subsequent release of inflammatory pain substances, leading to decreased excitability of inhibitory GABAergic interneurons and impaired their ability to inhibit peripheral pain signals. Ultimately, this establishes a detrimental feedback loop perpetuating pain sensitization.

and NCAN), as well as inflammatory pain substances (SP and IL-1β), in the contralateral mPFC region (Fig. 5D). The protein expression levels of BCAN and NCAN, SP and IL-1β were significantly decreased in the contralateral mPFC of rats in OA + ChABC group compared to those in OA group (*P<0.01*). However, compared with OA+ChABC group, there was no significant difference in the above indicators in OA+ChABC+HIIT group (*P>0.05*).

## Discussion

In this study, we established a correlation between changes in PNNs levels in the mPFC of rats and OA pain sensitization and elucidated the mechanism by which HIIT improves OA pain sensitization by regulating PNNs. We observed a significant decrease in the mechanical pain threshold on the operated side in rats with OA, which was accompanied by an increase in the level of PNNs in the contralateral mPFC. Degrading PNNs using ChABC significantly increased the mechanical pain threshold in rats with OA. Furthermore, HIIT significantly increased the mechanical pain threshold and reduced contralateral mPFC PNNs levels in rats with OA. However, HIIT after PNNs degradation did not further reduce PNNs levels or increase the mechanical pain threshold in rats with OA. Additionally, our study revealed significant upregulation of the inflammatory pain substances SP and IL-1β in the contralateral mPFC of rats with OA, and these levels could be reduced by HIIT. Notably, after ChABC-induced degradation of contralateral mPFC PNNs, HIIT did not reduce SP or IL-1β levels further, suggesting that HIIT may downregulate these inflammatory pain mediators by modulating PNNs levels.

Pain sensitization is a manifestation of maladaptive neuroplasticity and plays a crucial role in the development of chronic pain during OA [3-5,35]. PNNs, pivotal regulators of plasticity, significantly affect central neuroplasticity. Studies have demonstrated that targeting PNNs following spinal cord injury promotes axonal regeneration and enhances neuroplasticity [36]. Aberrant neuroplasticity resulting from PNNs defects has been implicated in various disorders, including dementia [37], anxiety disorders [38], and drug addiction [39]. The role of PNNs in chronic pain is currently under investigation. Research has revealed that chronic pain symptoms resulting from traumatic brain injury are associated with impaired cortical inhibition, which may rely on the protective effects of intact PNNs around $PV^+$ interneurons [19]. Multiple sclerosis-induced chronic neuropathic pain models exhibit disruptions in the connections between $PV^+$ interneurons in the primary somatosensory cortex (SSC) and significant reductions in PNNs, suggesting a link between PNNs and neuropathic pain [17]. Recent reports suggest that progressive establishment of PNNs occurs in the contralateral SSC, mPFC, and thalamic reticular nucleus during chronic inflammatory pain caused by unilateral injection of CFA into the hind paws of male mice. The degradation of PNNs induced by ChABC injection into the cortex significantly attenuates mechanical and thermal pain, suggesting the involvement of PNNs in pain sensitization caused by chronic inflammation [24]. However, the impact of alterations in PNNs levels in an OA model has not been previously documented. Pain sensitization can lead to the amplification of pain signals throughout the pain conduction axis, including the mPFC, which plays a pivotal role in pain processing [23]. We observed significant increases in the density of $WFA^+$ PNNs and the numbers of $WFA^+/PV^+$ cells in the contralateral mPFC of rats with pain sensitization 4 weeks after establishing the OA model through MIA injection into the knee joint. These findings are consistent with previous ones [24].

In our subsequent investigation, we examined alterations in key protein constituents of PNNs within the mPFC of rats with OA. We observed significant upregulation of the expression levels of BCAN and NCAN, which are crucial components of PNNs. BCAN is widely acknowledged as one of the most abundant CSPGs in the brain and serves as a fundamental constituent of PNNs [40]. Existing evidence suggests that BCAN may play a pivotal role in modulating cortical plasticity [41]. Similarly, NCAN, one of the primary proteoglycans found within PNNs, is believed to regulate critical neuronal developmental processes [42]. However, no significant changes in the expression of ACAN, another important component of PNNs, were observed. These findings indicate that chronic pain associated with OA leads to distinct changes in the expression of various constituents of PNNs. To further investigate the role of PNNs in OA pain sensitization, we used ChABC to enzymatically degrade PNNs in the contralateral mPFC. ChABC is a commonly used tool for studying the involvement of PNNs in the nervous system by specifically targeting and digesting glycosaminoglycans (GAGs) on PNNs [16]. As anticipated, the administration of ChABC resulted in analgesia and a significant reduction in the $WFA^+$ PNNs

density within the contralateral mPFC of rats with OA. Additionally, this treatment decreased the protein expression levels of BCAN and NCAN, highlighting the significance of distinct components within PNNs during OA pain sensitization. These findings suggest that further investigation of key protein constituents of PNNs can offer valuable insights into their contributions to OA pain sensitization.

Exercise has been shown to enhance neuroplasticity by modulating the levels of PNNs. Previous studies have shown that rats that were provided access to a running wheel exhibited reduced PNNs density in the hippocampus compared to sedentary rats, indicating improved plasticity [43]. HIIT, a specific form of exercise, has already been shown to ameliorate OA pain and functional impairment [21,44], although the underlying mechanisms remain unclear. This study investigated whether HIIT could ameliorate OA pain sensitization by regulating PNNs, and revealed that a 4-week HIIT regimen for rats with OA significantly increased the mechanical pain threshold on the operated side of knee joint. Additionally, we observed a notable decrease in the $WFA^+$ PNNs density in the contralateral mPFC, a significant reduction in the number of $WFA^+/PV^+$ cells, and a significant decrease in the protein expression levels of BCAN and NCAN. After administration of ChABC to the contralateral mPFC to degrade PNNs, followed by HIIT, neither additional reductions in the density of $WFA^+$ PNNs or the number of $WFA^+/PV^+$ cells within the contralateral mPFC region were observed, nor were there any further decreases in the protein expression levels of BCAN or NCAN. Similarly, a notable increase in the mechanical pain threshold was not observed in rats with OA, suggesting that HIIT may alleviate pain sensitization by modulating PNNs levels.

In this study, we also observed the effects of changes in PNNs levels in the mPFC of rats with OA on the inflammatory pain substances SP and IL-1β. Our findings revealed a significant increase in SP and IL-1β protein expression within the contralateral mPFC of rats with OA. However, HIIT significantly reduced the expression of these proteins. Additionally, injecting ChABC into the contralateral mPFC to digest PNNs significantly decreased SP and IL-1β protein expression. Subjecting rats with OA to HIIT after ChABC injection did not significantly change the levels of these indicators. Based on these findings, we hypothesize that the increased expression of SP and IL-1β proteins in the contralateral mPFC of OA rats may be associated with elevated levels of PNNs. However, as we have not thoroughly investigated the relationship between changes in PNNs and the release of inflammatory pain substances, nor their impact on OA pain sensitization, our current interpretations of the findings lack sufficient evidence. Therefore, further research is necessary to explore the impact of PNN alterations on the release of inflammatory pain substances and to determine whether HIIT can alleviate OA pain sensitization by reducing PNN levels, thereby decreasing the levels of inflammatory pain substances such as SP and IL-1β.

Finally, our study also observed the effect of HIIT on cartilage metabolism in OA rats. We found that HIIT increased the expression level of collagen II in the extracellular matrix of OA rats, while reducing the expression level of MMP-13, suggesting that HIIT may have a beneficial effect in promoting cartilage repair in OA rats.

It is important to acknowledge the limitations of our study. First, while our research demonstrated the efficacy of HIIT in reducing chronic pain in rats with OA, we only used a single HIIT protocol. Future investigations should explore different HIIT protocols to establish theoretical references for different populations with OA. Second, our study exclusively focused on the correlation between alterations in PNNs levels in the contralateral mPFC and chronic pain in rats with OA. Further investigation is warranted to comprehend the impact of changes in bilateral mPFC PNNs levels on $PV^+$ inhibitory interneuron excitability and their association with chronic pain in rats with OA.

In conclusion, this study elucidated the roles of PNNs in OA pain sensitization and highlighted the potential of HIIT to ameliorate OA pain sensitization by modulating PNNs within the mPFC. Our findings provide novel insights into the use of HIIT as an intervention strategy to alleviate OA-associated pain sensitization.

## Author contributions

Xiao Zhang and Xue-Ping Li conceived and designed the study. Xiao Zhang performed the experiments. Xiao Zhang wrote the manuscript. An-Liang Chen and Kai Cheng reviewed and edited the manuscript. Xiao Zhang collated and analyzed data. Fan-E Kong, Chang-Sheng Lin, and Zi-Qi Ye provided technical and logistic support. All authors read and approved the final version of the manuscript.

## Conflict of Interest

There is no conflict of interest.

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All animal experiments were performed following the Chinese Guidelines of Animal Care and Welfare, and the present study was approved by the Animal Care and Use Committee of China.

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