miR-3120/Hsc70 participates in forced swim stress-induced mechanical hyperalgesia in rats in an inflammatory state

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Abstract. The heat shock cognate 71 kDa protein (Hsc70) is a stress-inducible ATPase that can protect cells against harmful stimuli. Transient receptor potential vanilloid 1 (TRPV1) is a well-documented nociceptor. Notably, Hsc70 can inhibit TRPV1 expression and function, suggesting that Hsc70 may have pain regulation potential. However, the role of Hsc70 in stress-induced hyperalgesia remains unclear. In the present study, the participation of Hsc70 and its regulator microRNA (miR)-3120 were investigated in forced swim (FS) stress-induced mechanical hyperalgesia in rats in an inflammatory state. Complete Freund's adjuvant (CFA) hind paw injection was performed to induce inflammatory pain in rats (CFA rats). Furthermore, in FS + CFA rats, FS stress was performed for 3 days before CFA injection. The levels of Hsc70, miR-3120 and their downstream molecule TRPV1 were measured in the dorsal root ganglion (DRG) with western blotting, immunofluorescence, reverse transcription-quantitative polymerase chain reaction and fluorescence *in situ* hybridization. The results revealed that FS stress significantly exacerbated CFA-induced mechanical pain. Furthermore, CFA upregulated Hsc70 and TRPV1 expression, which was partially inhibited or further enhanced by FS stress, respectively. In FS + CFA rats, intrathecal injection of a lentiviral vector overexpressing Hsc70 (LV-Hsc70) could decrease TRPV1 expression and improve the mechanical pain. Additionally, the expression level of miR-3120, a regulator of Hsc70, was markedly upregulated on day 3 following FS stress. Finally, miR-3120 was identified to be colocalized with Hsc70 and expressed in all sizes of DRG neurons. In CFA rats, DRG injection of miR-3120 agomir to induce overexpression of miR-3120 resulted in similar TRPV1 expression and behavioral changes as those caused by FS stress, which were abolished in the presence of LV-Hsc70. These findings suggested that miR-3120/Hsc70 may participate in FS stress-induced mechanical hyperalgesia in rats in an inflammatory state, possibly via disinhibiting TRPV1 expression in the DRG neurons.

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Introduction

Depending on its nature, duration and intensity, stress has a distinctive effect on pain perception. Notably, acute or robust stress can induce analgesia; however, chronic or repeated exposure to various stressors can induce hyperalgesia or exacerbate existing pain, known as stress-induced hyperalgesia (SIH) (1). Various neural pathways participate in the development of SIH, including the cortex, amygdala, periaqueductal grey, rostral ventromedial medulla and spinal cord, involving diverse neurotransmitters and neuromodulatory systems (2). For example, glutamate content is increased at the spinal and supraspinal levels during stress to facilitate hyperalgesia if connected with ionotropic, but not metabotropic, glutamate receptors (3). Traumatic stress can promote hyperalgesia in rats by activating the corticotropin-releasing factor (CRF)/CRF receptor 1 pathway in the central amygdala (4), or via the interaction between microglia and neurons in the spinal dorsal horn (5). However, the molecular mechanisms underlying SIH remain unclear.

The heat shock cognate 71 kDa protein (Hsc70) is an ATPase that protects cells against various harmful stimuli. In particular, it is enriched in the nervous system and present at a high level in neuronal cell bodies (6). Hsc70 and its ATPase activity regulate transient receptor potential vanilloid 1 (TRPV1) expression and function via inhibiting ROCK phosphorylation at the TRPV1-S502 site in an Hsc70-dependent manner (7). Notably, TRPV1 is a well-documented nociceptor expressed in primary afferent dorsal root ganglion (DRG) neurons that participates in nociceptive perception (8). Increased TRPV1 expression is a characteristic of channel sensitization that results in hyperalgesia (9).

In addition, according to the dual-luciferase reporter assay and miRanda v1.0b prediction performed by Scott *et al* (10), microRNA (miRNA/miR)-3120 is an endogenous regulator of Hsc70. miRNAs are small non-coding RNA molecules that are comprised of 18-25 nucleotides, which recognize the 3' untranslated regions of target mRNA to modulate gene and protein expression, and are thus involved in a variety of biological processes (11). The etiological role of miRNAs in diverse painful conditions, such as migraine, fibromyalgia, visceral pain, and inflammatory or neuropathic pain, has been demonstrated (12). Although miRNAs have attracted great attention as diagnostic biomarkers and therapeutic targets, to the best of our knowledge, their participation in SIH has not been reported.

In the present study, complete Freund's adjuvant (CFA) hind paw injection was performed in rats to induce inflammatory pain (CFA rats). Furthermore, forced swim (FS) stress was performed for 3 days before CFA injection to evoke mechanical hyperalgesia (FS + CFA rats). Using this SIH rat model, the participation of Hsc70 and its regulator miR-3120 were investigated in FS stress-induced mechanical hyperalgesia in rats in an inflammatory state.

Materials and methods

Animals and ethics approval. The present study was approved by the Institutional Ethics Committee of Nanjing Medical University (approval no. 1706017; Nanjing, China). The present study was conducted using adult male Sprague-Dawley rats (age, 6-8 weeks; weight, 180-240 g; Qinglongshan Animal Center). Rats were housed individually with free access to food and water, and were acclimated to the environment with a temperature of 23-25˚C, a humidity of 40-60% and a 12-h light/dark cycle for at least 1 week before the experiment. After the experiment, all rats were euthanized by decapitation under deep anesthesia with 4-5% sevoflurane.

Animal models establishment. FS was induced in rats according to a previously described method (13). Briefly, in a cylinder (diameter, 30 cm; height, 50 cm) containing water at 24-26˚C to a height of 20 cm, the rats were forced to swim for 3 consecutive days, for 10 min on the first day and then for 20 min on the subsequent 2 days. After each FS session, the rats were carefully dried and rewarmed. Inflammatory pain was induced via a single injection of CFA (50 μl; MilliporeSigma) into the right hind paw. FS stress has previously been reported to increase pain-like behaviors in the hind paw (14). In the present study, FS stress-induced mechanical hyperalgesia in rats in an inflammatory state was established using FS stress for 3 days, followed by a single CFA injection. Control rats were injected with 50 μl deionized water. To verify the efficacy of FS stress-induced mechanical hyperalgesia in rats in an inflammatory state, 6 rats were used in each group.

ELISA detection for serum corticosterone. For serum corticosterone detection, 300 μl blood was collected from the tail vein under 2-3% sevoflurane anesthesia 2 h after FS stress, and the plasma was separated and stored at -80˚C until the ELISA was performed. A commercially available ELISA kit (cat. no. ab108821; Abcam) was used to measure serum corticosterone levels according to the manufacturer's instructions. The OD value was acquired at 450 nm using a multi-function microplate reader (MD Spectramac M3; Molecular Devices, LLC). Different rat groups were measured across days 1-3, with three rats assessed at each time point. For control rats, the blood was collected at the approximately same time as for rats in the other groups.

Pain behavioral test. Mechanical pain was examined using von-Frey filaments. Briefly, the rats were individually placed in a transparent Plexiglass chamber for 30 min. Thereafter, a series of von Frey filaments (0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0 and 15 g; Danmic Global, LLC) were vertically applied to the central plantar of the hind paw to evoke a flinch response using an up-down method (15). The paw withdrawal threshold was detected five times with an interval of 5 min, and the mean value of the last three results was calculated as the mechanical withdrawal threshold.

Western blot analysis. Hsc70 and TRPV1 expression levels in the DRG were examined by western blot, as previously described (16). After the pain behavioral test, the rats were sacrificed by decapitation under 4-5% sevoflurane anesthesia and L4-5 DRG was collected for western blot analysis. Briefly, the DRG was separated and homogenized in RIPA lysis buffer (cat. no. bl504a; Biosharp Life Sciences), and the resulting supernatant was collected. After protein quantification using the Bradford assay, 20 μg proteins were separated by SDS-PAGE 8-12% on gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature, and then incubated with primary antibodies against Hsc70 (1:500; cat. no. ab51052), TRPV1 (1:200; cat. no. ab305299) overnight at 4˚C, and GAPDH (1:1,000; cat. no. ab8245) (all from Abcam) was used as an internal control. After washing in Tris-buffered saline-0.05% Tween three times, the membranes were incubated with horseradish peroxidase-labeled immunoglobulin G (1:5,000; cat. no. bl003A; Biosharp Life Sciences) for 1 h at room temperature. The bands were detected using a luminescent imaging system (G:BOX Chemi XR5; Syngene) and the blots were analyzed using ImageJ software (v. 1.53a; National Institutes of Health).

Immunofluorescence (IF) analysis. In the DRG, Hsc70 and TRPV1 expression levels were also detected by IF analysis. After sacrifice, the rats were transcardially perfused with 4% paraformaldehyde. The L4-5 DRG was removed, fixed in the same fixative at 4˚C for 6 h, and thereafter embedded in paraffin. For IF staining, the DRG tissue (3 μm) was mounted on glass slides. After sequential dewaxing, hydration and microwave antigen retrieval in sodium citrate for 20 min, the sections were blocked with 5% bovine serum albumin (cat. no. 4240GR500; Biofroxx; neoFroxx GmbH) at 37˚C for 1 h. After overnight incubation with a rabbit antibody against Hsc70 (1:200; cat. no. ab51052) or a mouse antibody against TRPV1 (1:200; cat. no. ab203103) (both from Abcam) at 4˚C, the sections were incubated with fluorescein isothiocyanate-conjugated (1:200; cat. no. BL033A; Biosharp Life Sciences) or tetramethylrhodamine-conjugated (1:100; cat. no. 115-025-003; Jackson ImmunoResearch Europe

Table I. Primer sequences.

Gene	Forward, 5'-3'	Reverse, 5'-3'
miR-3120	CGCGCACAGCAAGTGTAGA	AGTGCAGGGTCCGAGGTATT
U6	CTCGCTTCGGCAGCACA	TGGTGTCGTGGAGTCG
Hsc70	TACCCGTGCTCGATTTGAGG	GAACCACCCACCAGGACAAT
GAPDH	CGGCAAGTTCAACGGCACAGT	CGCTCCTGGAAGATGGTGATGG

miR-3120, microRNA-3120; Hsc70, heat shock cognate 71 kDa protein.

Ltd.) secondary antibodies for 1 h at 37˚C. Nuclear DNA was labelled with DAPI. The obtained IF sections were scanned and stored using an OlyVIA system (Olympus VS200; Olympus Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for miR-3120 and Hsc70. RT-qPCR detection of miR-3120 and Hsc70 expression in the DRG was performed. Briefly, total RNA was extracted from the DRG with ice-cold TRIzol® reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, RNA concentration and purity were determined using an ultraviolet photometer (Shimadzu UV-2450; Shimadzu Corporation) at 260 and 280 nm; a result of 1.8-2.1 was considered an appropriate purity. Using 2 μg total RNA as a template, cDNA was synthesized using a reverse transcriptase reagent kit (cat. no. RR036B; Takara Bio, Inc.) according to the manufacturer's instructions. qPCR amplification was performed using the One Step TB Green PrimeScript RT-PCR Kit II (cat. no. RR086B; Takara Bio, Inc.). The cycling conditions were: 95˚C for 5 min, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ method was used to calculate the gene expression relative to the reference gene (17).

For detection of miR-3120, Bulge-loop™ miRNA qPCR Primer Sets specific for miR-3120 were designed by Nanjing KeyGen Biotech Co., Ltd. The primer sequences for miR-3120 and its internal control U6, as well as for Hsc70 and its internal control GAPDH, which were used for qPCR, are shown in Table I. To examine stress-induced changes in miR-3120 expression, the same rats used for serum corticosterone detection were assessed. Briefly, three rats were used to detect serum corticosterone levels at each time point, after which they were sacrificed, the DRG was dissected and miR-3120 expression was detected.

miR-3120 agomir production and DRG injection. Cy3-labeled miR-3120 agomir, which encodes the red fluorescence protein Cy3 and miR-3120, as well as its negative control (NC; Cy3-labeled agomir NC), were produced by Nanjing KeyGen Biotech Co., Ltd. For the DRG injection, the paraspinal muscles were carefully separated along the vertebrae under 2-3% sevoflurane anesthesia and 4 mg/kg lidocaine was intraincisionally injected. Part of the bone covering the right L5 DRG was then removed to completely expose the ganglion. miR-3120 agomir or agomir NC (0.5 nM dissolved in RNAase-free deionized water to 5 μl) was slowly injected into the L5 DRG using a Hamilton microliter syringe with a 30-gauge needle 3 days before CFA injection in rats in the CFA + miR-3120 agomir or CFA + agomir NC groups.

Fluorescence in situ hybridization (FISH) for miR-3120. Cellular distribution of miR-3120 in the DRG was examined using FISH according to a previously described method (18). Briefly, paraffin-embedded DRG slides were prepared using the same method as that described for IF analysis. After sequential dewaxing, hydration and permeabilized with 0.4% Triton X-100 for 15 min at room temperature, the slides were washed twice with 2X saline sodium citrate (SSC). The oligonucleotide probe was denatured for 5 min at 75˚C in a water bath, and the slides were incubated with the pre-hybridization solution (cat. no. AR0152; Boster Biological Technology) at 42˚C for 30 min, followed by 40 nM probe at 42˚C overnight. After sequential washing with 2, 1, 0.5 and 0.1X SSC, the slides were counterstained with Hoechst 33258 (cat. no. C1017; Beyotime Institute of Biotechnology). The sections were scanned and stored using the OlyVIA system (Olympus VS200) with the required images exported when necessary. In addition, the cellular distribution of miR-3120 and Hsc70 was examined in the DRG using FISH and IF analysis, respectively. After FISH and IF staining, the colocalization, as well as the size of miR-3120- and Hsc70-labeled cells was analyzed using ImageJ software.

Lentiviral vector construction and production. Full-length Hsc70 (Gene ID: 24468; Transcript: NM_024351.2) or scramble oligonucleotides were subcloned into the GV280 lentiviral vector (Nanjing KeyGen Biotech Co., Ltd.) to construct a lentiviral vector overexpressing Hsc70 (LV-Hsc70; $1x10^8$ TU/ml) and an LV-scramble, respectively. According to a previously reported method (19), lentiviral vector (10 μl) was intrathecally microinjected into the L5-6 intervertebral space of rats through the skin using a 30-gauge needle attached to a Hamilton microliter syringe under 2-3% sevoflurane anesthesia 1 week before CFA injection in the CFA + agomir + LV-Hsc70 and CFA + agomir + LV-scramble groups.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8.0 software (Dotmatics). Data are shown as the mean ± SEM. One-way ANOVA was used to assess the changes in the mechanical withdrawal threshold at each time point among the groups. In addition, one-way ANOVA was used to assess differences in the results of ELISA, western blotting, IF analysis and RT-qPCR among the groups. If a significant difference was observed, the Bonferroni post hoc

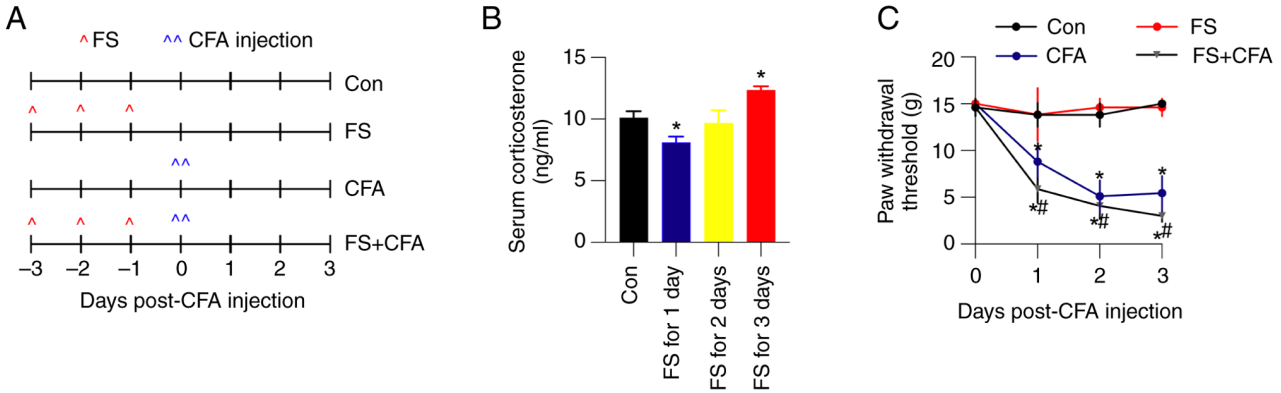

Figure 1. FS stress increases serum corticosterone levels and exacerbates CFA-induced mechanical pain in rats. (A) Schematic diagram of CFA-induced mechanical pain and FS stress-induced mechanical hyperalgesia in rats. (B) Serum corticosterone levels in control and stressed rats was examined with ELISA (n=3 rats/group at each time point). (C) Mechanical withdrawal threshold in control, FS, CFA and FS + CFA rats 3 days following CFA injection (n=6 rats/group). Data are presented as the mean ± SEM. *P<0.05 vs. Con; $^{\#}$P<0.05 vs. CFA. Con, control; CFA, complete Freund's adjuvant; FS, forced swim.

test was applied. P<0.05 was considered to indicate a statistically significant difference.

Results

FS stress increases serum corticosterone and exacerbates CFA-induced mechanical pain in rats. In the present study, CFA hind paw injection was performed in rats to induce inflammatory pain (CFA rats). In addition, FS stress was performed for 3 days before CFA injection to evoke mechanical hyperalgesia to establish an SIH rat model (FS + CFA rats). A schematic diagram of the model is presented in Fig. 1A. To verify the efficiency of FS stress in rats, serum corticosterone levels were examined using ELISA. In control rats, the average serum corticosterone level was 10.1±0.5 ng/ml. After 3 days of FS stress, the average corticosterone concentration significantly increased to 12.3±0.3 ng/ml (Fig. 1B). Moreover, CFA hind paw injection quickly decreased the paw withdrawal threshold, which was further exacerbated by 3 days of FS stress (Fig. 1C). All of these observations indicated the successful establishment of FS stress-induced mechanical hyperalgesia in rats in an inflammatory state.

CFA upregulates Hsc70 and TRPV1 expression in the DRG, which is partially inhibited or further enhanced by FS stress. Hsc70 participates in CFA-induced inflammatory pain (6); however, its involvement in FS stress-induced mechanical hyperalgesia remains largely unknown. Therefore, the expression of Hsc70 in the DRG was detected using western blot and IF analysis. The results showed that Hsc70 protein expression was increased by ~1.9-fold in CFA; however, this increase was partially inhibited in FS + CFA rats (Fig. 2A). Consistent with the results of western blot, the results of IF analysis suggested a similar change in Hsc70 expression (Fig. 2B). However, FS stress alone did not significantly influence Hsc70 expression.

TRPV1 protein expression was increased by ~1.6-fold in CFA rats, which was further increased to ~2-fold after 3 days of FS stress (Fig. 2C). IF analysis exhibited a similar expression change (Fig. 2D). Notably, TPRV1 is a well-known nociceptor in pain perception. Moreover, to verify the etiological role of Hsc70 in FS stress-induced mechanical hyperalgesia, LV-Hsc70 was intrathecally injected into FS + CFA rats to increase the expression of Hsc70 the day before FS. The efficiency of intrathecal LV-Hsc70 was verified using RT-qPCR after 1 week (Fig. 2E). In the presence of intrathecal LV-Hsc70, TRPV1 expression in the DRG was partially inhibited (Fig. 2F) and the mechanical withdrawal threshold was increased (Fig. 2G) compared with that in the FS + CFA group. These results suggested that Hsc70 and TRPV1 may have an essential role in FS stress-induced mechanical hyperalgesia in rats in an inflammatory state.

FS stress upregulates miR-3120 expression in the DRG. As an endogenous ligand of Hsc70, miR-3120 is located in the neuronal cell body, and it targets and inhibits Hsc70 (10). To explore the potential regulatory role of miR-3120 for Hsc70, miR-3120 expression was examined by RT-qPCR in the DRG following FS stress. The results showed that 3 days of FS stress significantly upregulated the expression levels of miR-3120 by ~2.6-fold (Fig. 3A). Moreover, FS stress for 1 day transiently downregulated miR-3120 expression. This downregulation was consistent with that observed for serum corticosterone; however, the underlying reason remains unclear.

The cellular distribution of miR-3120 and Hsc70 was then examined in the DRG using FISH and IF analysis, respectively. After double fluorescence staining, the colocalization of miR-3120 and Hsc70 was analyzed, as well as the diameter of the labeled cells. miR-3120 and Hsc70 were both expressed on the cell surface and in the cytoplasm, and a number of miR-3120 and Hsc70 double-labelled neurons were observed (Fig. 3B). Cell size distribution histograms for miR-3120 (red) and Hsc70 (green) showed that more than half of the miR-3120 and Hsc70 double-labeled neurons were medium and large-sized neurons (cross-sectional area >600 μm^2) (Fig. 3C). These results indicated that miR-3120 and Hsc70 may act on the same population of neurons.

Fluorescence detection and RT-qPCR verify the efficiency of miR-3120 agomir injection. To explore the participation of miR-3120 in FS stress-induced mechanical hyperalgesia, miR-3120 agomir was injected into the DRG in CFA rats. Agomirs are specially labeled and chemically

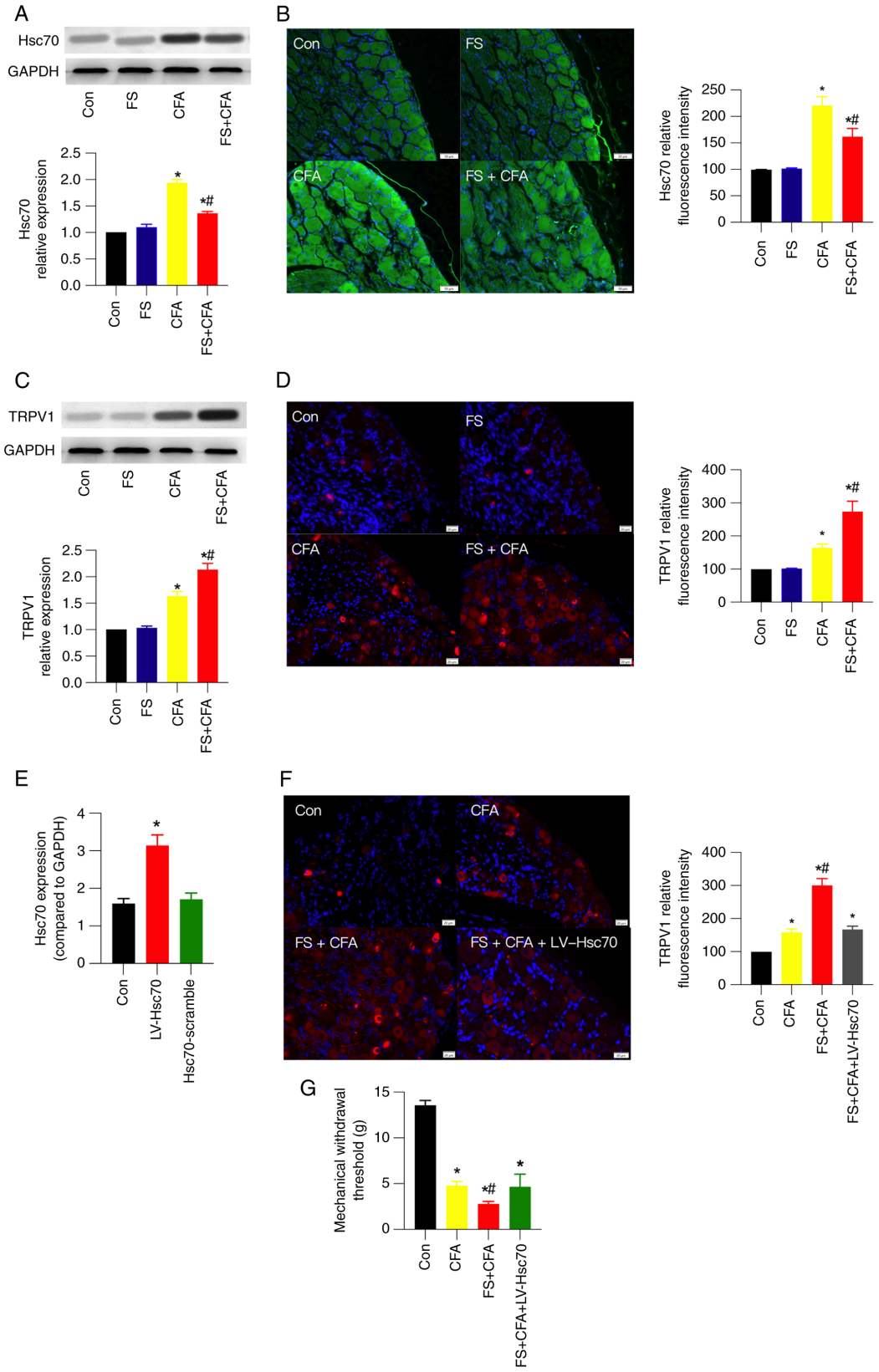

Figure 2. CFA upregulates Hsc70 and TRPV1 expression in the DRG, which is partially inhibited or further enhanced by FS stress, respectively. (A) Western blotting images and their statistical analysis, and (B) IF images and their statistical analysis for Hsc70 in control, FS, CFA and FS + CFA rats (n=3 rats/group). Scale bar, 50 μm. (C) Western blotting images and their statistical analysis, and (D) IF images and their statistical analysis for TRPV1 expression in the four groups. (n=3 rats/group). Scale bar, 20 μm. To verify the etiological role of Hsc70 in FS stress-induced mechanical hyperalgesia, LV-Hsc70 was intrathecally injected into FS + CFA rats to increase its expression the day before FS. (E) Reverse transcription-quantitative PCR results showed that intrathecal injection of LV-Hsc70, but not its scramble, successfully increased Hsc70 expression in the DRG. (F) IF images and corresponding statistical analysis of TRPV1 expression in the control, CFA, FS + CFA and FS + CFA + LV-Hsc70 rats. Scale bar, 20 μm. (G) Mechanical withdrawal threshold in rats in the four groups (n=6 rats/group). For statistical analysis of IF images, 4-5 images from 2-3 rats were analyzed in each group. Data are presented as the mean ± SEM. *P<0.05 vs. Con; $^{\#}$P<0.05 vs. CFA. Con, control; CFA, complete Freund's adjuvant; DRG, dorsal root ganglion; FS, forced swim; Hsc70, heat shock cognate 71 kDa protein; LV, lentivirus; TRPV1, transient receptor potential vanilloid 1.

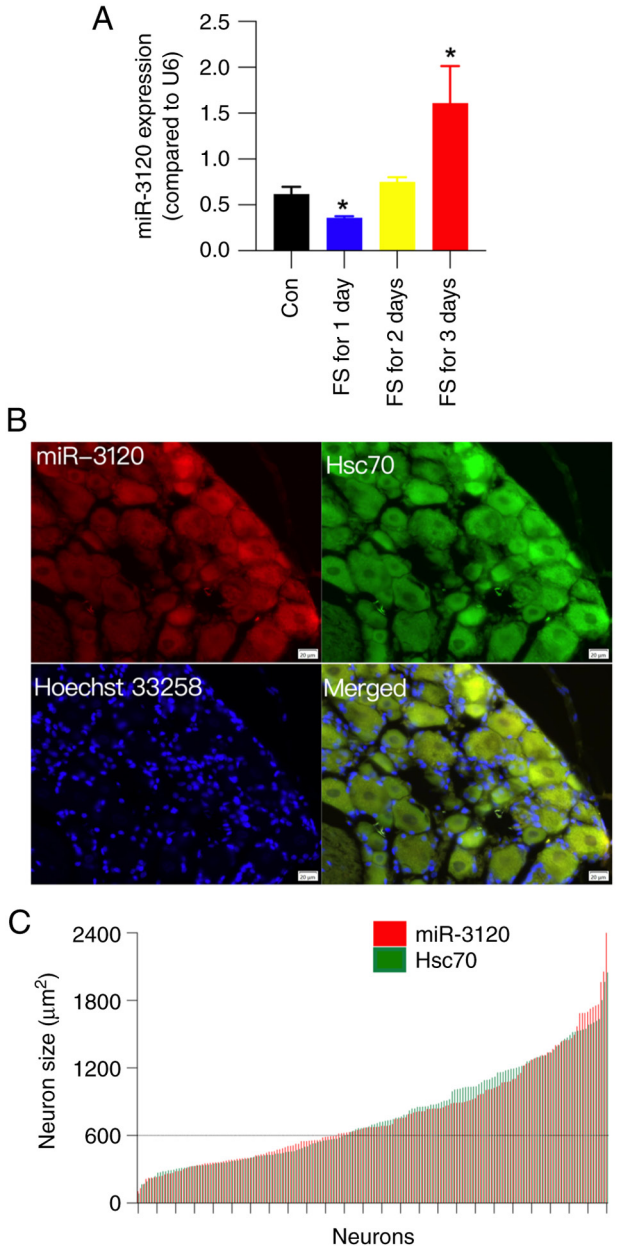

Figure 3. FS stress upregulates miR-3120 expression in the DRG. (A) miR-3120 expression in Con and FS rats was examined with reverse transcription-quantitative PCR (n=3-4 rats/group). (B) miR-3120 and Hsc70 expression in control rats was detected using fluorescence *in situ* hybridization and immunofluorescence analysis, respectively. Representative double-fluorescence staining images of miR-3120 and Hsc70 in the DRG are shown. Scale bar, 20 μm. (C) Cell size distribution for miR-3120 and Hsc70 double-labeled neurons was quantitatively analyzed. For statistical analysis, ~200 neurons in four sections from two rats were analyzed. Data are presented as the mean ± SEM. *P<0.05 vs. Con. Con, control; DRG, dorsal root ganglion; FS, forced swim; Hsc70, heat shock cognate 71 kDa protein; miR-3120, microRNA-3120.

modified double-stranded small RNA molecules that simulate endogenous miRNAs to regulate the biological functions of target genes. Compared with miRNA mimics, agomirs can enrich in target cells, presenting higher stability *in vivo* and resulting in more effective interference (20).

The efficiency of the miR-3120 agomir was examined using fluorescence detection and RT-qPCR. The results showed that the injection of Cy3-labeled miR-3120 agomir encoding both the red fluorescence protein Cy3 and miR-3120 into the L5 DRG induced red fluorescence in all sizes of DRG neurons as early as 48 h after injection (Fig. 4A). Although DRG injection of agomir NC also presented red fluorescence (Fig. 4A), RT-qPCR showed that miR-3120 agomir, but not agomir NC, significantly increased the expression levels of miR-3120 at 72 h after DRG injection compared with those in the control group (Fig. 4B), collectively verifying the efficiency of miR-3120 agomir after DRG injection.

DRG injection of miR-3120 agomir upregulates TRPV1 expression and induces mechanical hyperalgesia in CFA rats. After verifying the efficiency of DRG injection of miR-3120 agomir, the miR-3120 agomir or its NC was injected into the DRG 3 days before CFA injection. Western blotting showed that miR-3120 agomir, but not its NC, significantly upregulated TRPV1 expression by ~2-fold in CFA rats (Fig. 5A). Meanwhile, miR-3120 agomir significantly enhanced CFA-induced mechanical pain, which was similar to FS stress-induced behavioral changes detected in CFA rats (Fig. 5B). These results suggested that miR-3120 may participate in FS stress-induced mechanical hyperalgesia.

miR-3120 agomir-induced TRPV1 expression and mechanical hyperalgesia in CFA rats are abolished in the presence of Hsc70 overexpression. To further investigate whether Hsc70 is involved in miR-3120 agomir-induced TRPV1 expression and behavioral changes, miR-3120 agomir and LV-Hsc70 were injected into the DRG 3 days and intrathecally injected 1 week before CFA injection, respectively. As shown by western blotting and behavioral testing, miR-3120 agomir-induced TRPV1 expression and mechanical hyperalgesia in CFA rats were significantly abolished in the presence of intrathecal injection of LV-Hsc70 but not LV-scramble (Fig. 6A and B).

Discussion

The results of the present study suggested that miR-3120/Hsc70 may participate in FS stress-induced mechanical hyperalgesia in rats in an inflammatory state, possibly via disinhibiting TRPV1 expression in the DRG neurons. In CFA rats, upregulated Hsc70 may inhibit TRPV1 expression and work as a protective mechanism to control pain signals.

Hsc70 and TRPV1 expression was increased in the DRG after CFA injection. As a member of the Hsp70 family, Hsc70 is a type of constitutively expressed molecular cognate protein that is essential for a number of cellular functions. Notably, Hsc70 acts as a clathrin-uncoating ATPase during clathrin-mediated endocytosis, maintaining protein homeostasis, antigen processing and presentation, and importing proteins into organelles or cellular compartments (21).

In the DRG, exogenous Hsc70 has been shown to prevent injury-induced death of nearly all sensory neurons in neonatal mice undergoing bilateral transection of the sciatic nerve (22). Inhibition of Hsp90 to upregulate Hsp70 may protect against glucose-induced embryonic DRG neuron death, improve nerve conduction velocity and sensory deficits, and reverse sensory hypoalgesia in diabetic mice (23). However, whether Hsc70 in the DRG has a protective role in inflammatory pain has seldom been reported. Iftinca *et al* (7) detected increased

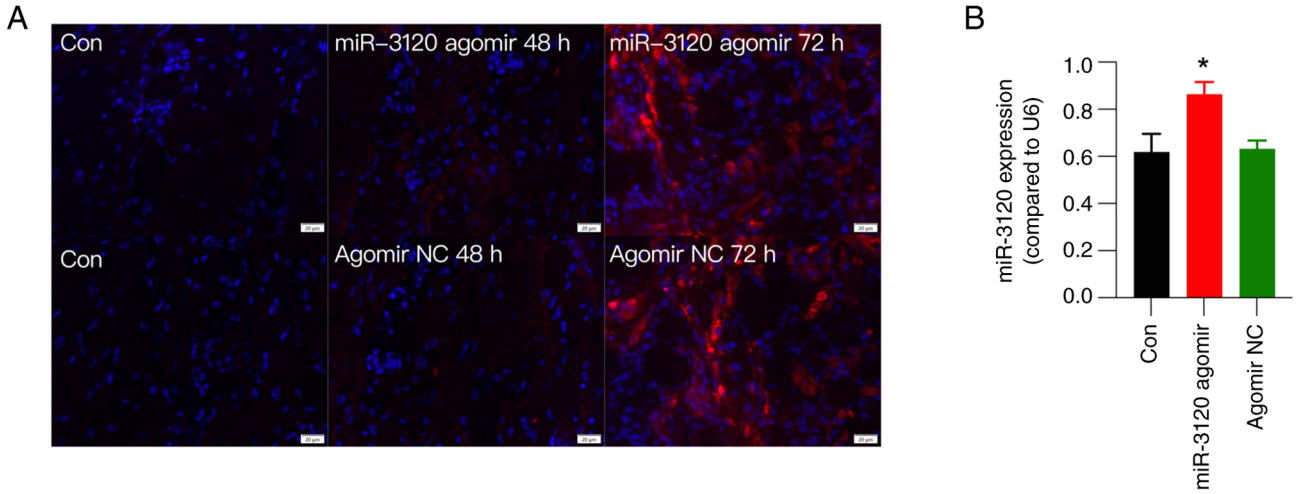

Figure 4. Fluorescence detection and RT-qPCR verify the efficiency of the miR-3120 agomir. (A) Fluorescence images of the DRG at 48 or 72 h after miR-3120 agomir or agomir NC injection. Scale bar, 20 μm. (B) RT-qPCR examined miR-3120 expression levels at 72 h after DRG injection (n=3 rats/group). *P<0.05 vs. Con. Con, control; DRG, dorsal root ganglion; miR-3120, microRNA-3120; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.

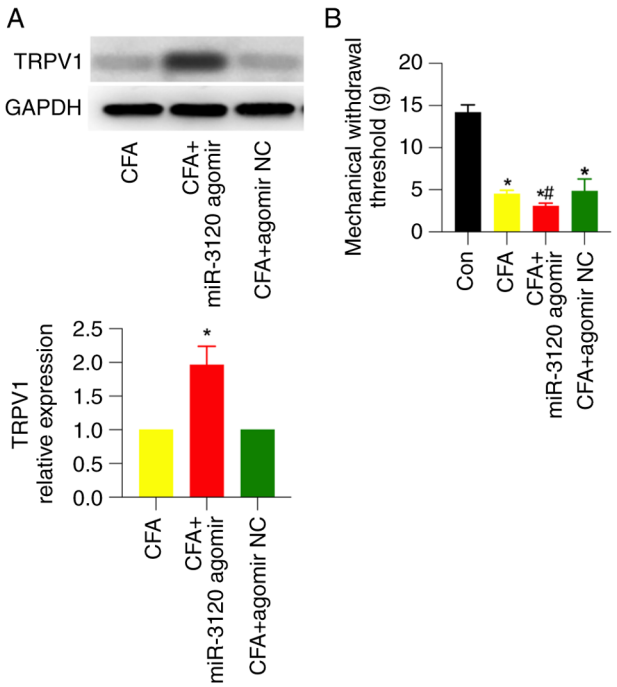

Figure 5. DRG injection of miR-3120 agomir upregulates TRPV1 expression and induces mechanical hyperalgesia in CFA rats. miR-3120 agomir or its NC was injected into the DRG 3 days before CFA injection. (A) Representative western blot images and corresponding statistical analysis of TRPV1 expression in the DRG 3 days post-CFA injection in CFA, CFA + miR-3120 agomir and CFA + agomir NC rats (n=3 rats/group). *P<0.05 vs. CFA. (B) Mechanical withdrawal threshold of rats in the four groups 3 days after CFA injection (n=6 rats/group). *P<0.05 vs. Con; #P<0.05 vs. CFA. Data are shown as mean ± SEM. CFA, complete Freund's adjuvant; DRG, dorsal root ganglion; miR-3120, microRNA-3120; NC, negative control; TRPV1, transient receptor potential vanilloid 1.

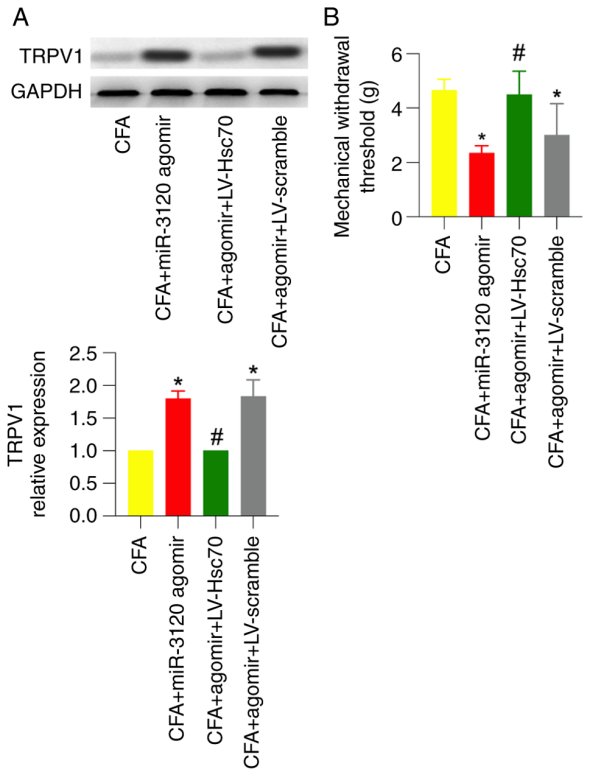

Figure 6. miR-3120 agomir-induced TRPV1 expression and mechanical hyperalgesia in CFA rats are abolished in the presence of Hsc70 overexpression. miR-3120 agomir and LV-Hsc70 were DRG or intrathecally injected at 3 days and 1 week before CFA injection, respectively. (A) Representative western blot images and corresponding statistical analysis of TRPV1 expression in the DRG 3 days after CFA injection in CFA, CFA + miR-3120 agomir, CFA + miR-3120 agomir + LV-Hsc70 and CFA + miR-3120 agomir + LV-scramble rats (n=3 rats/group). (B) Mechanical withdrawal threshold in the four groups 3 days after CFA injection (n=6 rats/group). Data are presented as the mean ± SEM. *P<0.05 vs. CFA; #P<0.05 vs. CFA + miR-3120 agomir. CFA, complete Freund's adjuvant; DRG, dorsal root ganglion; Hsc70, heat shock cognate 71 kDa protein; LV, lentivirus; miR-3120, microRNA-3120; TRPV1, transient receptor potential vanilloid 1.

Hsc70 protein levels in the DRG 3 days post-CFA hind paw injection. The increased Hsc70 was proposed to inhibit TRPV1 expression via inhibiting ROCK phosphorylation of the TRPV1-S502 site in an Hsc70-dependent manner, thus forming a functional link between Hsc70 and TRPV1. The present also observed an increase in TRPV1 expression in CFA rats in the present study, which is consistent with previous reports (24,25).

The present study focused on the possible contribution of Hsc70 and TRPV1 to FS stress-induced mechanical hyperalgesia in CFA rats. As shown by western blotting and IF analysis, FS stress inhibited CFA-induced upregulation of Hsc70 expression. Sato *et al* (26) reported that chronic stress-related dexamethasone significantly decreased lysosomal Hsc70 expression in human cell lines and primary cultured rat neurons, indicating that chronic stress-related corticosteroid secretion may alter Hsc70 expression. In the present study, FS stress for 3 days markedly increased serum corticosterone levels, indicating the successful establishment of a chronic stress model. Such FS stress partially inhibited the increase in Hsc70 expression in CFA rats, which was accompanied by a further increase in TRPV1 expression and more severe pain in CFA rats. As a type of capsaicin-sensitive nociceptor, TRPV1 has been shown to protect against stress-induced mechanical hyperalgesia, potentially by influencing neuronal plasticity (27). Following stress-induced visceral hyperalgesia, increased TRPV1 expression in the DRG was observed, suggesting the stress sensitivity of TRPV1 nociceptors (28).

FS stress for 3 days increased the expression of miR-3120, which has been reported to participate in synapse vesicle function and neuronal plasticity (10,29). Numerous miRNAs in the DRG have been suggested to participate in CFA-related inflammatory pain. For example, decreased miR-485-5p and miR-134 expression have been shown to contribute to CFA-induced inflammatory pain by upregulating ASIC1 and MOR1 expression, respectively (30,31). Moreover, CFA-related inflammation can reduce miR-1, -16 and -206 expression in the DRG (32), indicating that miRNAs may participate in the nociceptive process following noxious stimuli. However, to the best of our knowledge, the role of miR-3120 in pain regulation has not been reported. According to the dual-luciferase reporter assay and miRanda v1.0b prediction performed by Scott *et al* (10), miR-3120 may target and inhibit Hsc70, playing an essential role in regulating the constitutive level of Hsc70. In the present study, DRG injection of miR-3120 agomir significantly increased miR-3120 and TRPV1 expression, and exacerbated CFA-induced inflammatory pain, replicating FS stress-induced inflammatory hyperalgesia in CFA rats.

To explore the potential role of Hsc70 in miR-3120-induced TRPV1 expression and behavioral changes, miR-3120 and Hsc70 were double stained in the DRG. Both miR-3120 and Hsc70 were found on the cell surface and in the cytoplasm. Cell size distribution analysis for miR-3120 and Hsc70 showed that in normal rats, more than half of the miR-3120 and Hsc70 double-labeled neurons were large. Small-, medium- and large-sized neurons in the DRG were assumed to be C-fiber-, Aδ- and Aβ-related sensory neurons, respectively. Under physiological conditions, small- and medium-sized neurons have been suggested to mediate nociceptive behaviors in the DRG; however, Aβ primary afferent neurons have recently been proposed to become hyperexcitable in response to painful stressors (33,34). In the present study, more than half of the miR-3120 and Hsc70 double-labeled neurons were observed to be medium and large-sized neurons, suggesting that Aδ and Aβ primary afferent neurons and their fibers may play an essential role in FS stress-related mechanical hyperalgesia. However, the present study did not examine the distribution of miR-3120- or Hsc70-expressing neurons after CFA or FS stress. Further research is required to assess this.

LV-Hsc70 was intrathecally injected 1 week before CFA injection to induce the overexpression of Hsc70 in rats. As expected, miR-3120 agomir-induced TRPV1 upregulation and mechanical hyperalgesia in CFA rats were abolished in the presence of Hsc70 overexpression. This result suggested the involvement of Hsc70 in miR-3120 agomir-induced TRPV1 upregulation. Increased Hsc70 expression may control TRPV1 expression and act as a protective mechanism to control pain signals mediated by TRPV1 activation in CFA rats. Numerous studies have suggested a protective role of Hsc70 in various neurological diseases, including Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis and multiple system atrophy (35,36). Hsc70 translocates to synapse-enriched areas in the cerebral cortex to refold denatured proteins after thermal stress (6). In addition, Hsc70 directly participates in cell survival during neurulation, and acts as an intrinsic protector of neuroepithelial and neural precursor cells (37). Exogenous Hsc70 incubation prior to oxidative injury has been shown to protect motor neurons from oxidative stress injury (38). Furthermore, intracerebroventricular administration of Hsp70/Hsc70 can reduce the severity of chemically induced seizures (39). To the best of our knowledge, the present study is the first to suggest the potential protective role of Hsc70 in CFA-induced inflammatory pain.

In addition to the miR-3120/Hsc70 mechanism, the underlying mechanisms for FS stress-induced inflammatory hyperalgesia have not been elucidated. The role of the gut microbiota, neuronal plasticity in the brain, dysfunction of the descending pain regulatory system and excitatory-inhibitory neurotransmission imbalance at the spinal level have been suggested to participate in FS stress-related hyperalgesia (40,41). Furthermore, the functional study of miR-3120, Hsc70 and TRPV1 in SIH remains to be elucidated. One specific finding of the present study was that serum corticosterone levels and miR-3120 expression in the DRG were transiently decreased following FS stress for 1 day. Considering the etiological role of increased serum corticosterone and miR-3120 in SIH, such a transient decrease may contribute to another contrasting pain phenomenon, stress-induced analgesia. However, this was not explored in the present study and thus requires further verification.

In conclusion, the results of the present study suggested that miR-3120/Hsc70 may participate in FS stress-induced mechanical hyperalgesia in rats in an inflammatory state, possibly via disinhibiting TRPV1 expression in the DRG neurons.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW and SF designed the study and provided funding support. SX and SL performed the behavioral test and were major contributors in writing the manuscript. JY, RL and MM performed all biological and histological examinations in this study, collected original data and performed statistical analysis. XW and SF confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of Nanjing Medical University (approval no. 1706017).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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