Activation of SIRT1 Alleviates Mitochondrial Dysfunction in The Trigeminal Nucleus Caudalis in a Rat Model of Chronic Migraine

jie liang
Chongqing Medical University First Affiliated Hospital
xue zhou
Chongqing Medical University First Affiliated Hospital
jiang wang
Chongqing Medical University First Affiliated Hospital
zhaoyang fei
Chongqing Medical University First Affiliated Hospital
guangcheng qin
Chongqing Medical University First Affiliated Hospital
dunke zhang
Chongqing Medical University First Affiliated Hospital
jiying zhou
Chongqing Medical University First Affiliated Hospital
Li-Xue Chen (chenlixue@hospital.cqmu.edu.cn)
Chongqing Medical University First Affiliated Hospital https://orcid.org/0000-0001-8213-8828

Research article

Keywords: Chronic migraine.Mitochondrial dysfunction. SIRT1.Trigeminal nucleus caudalis

Posted Date: January 28th, 2020

DOI: https://doi.org/10.21203/rs.2.21979/v1

Abstract

Background: The mechanism of chronic migraine (CM) is still unclear and mitochondrial dysfunction plays a possible role in migraine pathophysiology. Silent information regulator 1 (SIRT1) plays a vital role in mitochondrial dysfunction in many diseases, but there is no information about SIRT1 in CM.The aim of this study was to explore the role of SIRT1 in mitochondrial dysfunction in CM. Methods A rat model was established through repeated dural infusions of inflammatory soup (IS) for seven days to simulate CM attacks. Cutaneous hyperalgesia caused by the repeated infusions of IS was detected using the von Frey test. Then, we detected SIRT1 expression in the trigeminal nucleus caudalis (TNC). To explore the effect of SIRT1 on mitochondrial dysfunction in CM rats, we examined whether SRT1720, an activator of SIRT1, altered mitochondrial dysfunction in CM rats. Results Repeated infusions of IS resulted in cutaneous hyperalgesia accompanied bydownregulation of SIRT1.SRT1720 significantly alleviated the cutaneous hyperalgesia induced by repeated infusions of IS. Furthermore, activation of SIRT1 markedly increased the expression of peroxisome proliferator-activated receptor gamma-coactivator 1-alpha(PGC-1α), transcription factor A (TFAM), nuclear respiratory factor 1 (NRF-1), and nuclear respiratory factor 2(NRF-2) mitochondrial DNA (mtDNA) and increased the ATP content and mitochondrial membrane potential. Conclusions Our results indicate that SIRT1 may have an effect on mitochondrial dysfunction in CM rats. Activation of SIRT1 has a protective effect on mitochondrial function in CM rats.

Background

Migraine is an incapacecitating and complex neurovascular disorder that affects the quality of both the work and life of patients, and migraine is one of the most prevalent and disabling medical illnesses in the world[1, 2]. According to the number of headache attacks per month, migraine can be classified as episodic migraine (EM) or chronic migraine (CM)[3]. The prevalence of CM is 1–3% in the general population, and it is estimated that approximately 3% of EM cases evolve into CM per year[4, 5].

However, the pathophysiology of migraine remains unclear. Due to the high dependence of neural activity on energy, aberrant mitochondrial morphology and function can cause various neurological diseases[6, 7]. Previous studies have suggested that mitochondrial dysfunction plays a possible role in migraine pathophysiology[8, 9]. It has been reported that mitochondrial function is often impaired in patients with migraine[10]. Furthermore, a previous study found abnormal mitochondrial dynamics and impaired mitochondrial biogenesis in trigeminal ganglion neurons in a repeated IS infusion rat model of migraine[11].However, there have been few studies about mitochondrial dysfunction in the trigeminal nucleus caudalis (TNC) in CM, and there have also been few investigations on the signaling pathway of mitochondrial dysfunction in CM.Therefore, further elucidation of the specific mechanism of mitochondrial dysfunction in the CM circuit will facilitate our understanding of the pathophysiology of CM and promote the development of new therapeutics.

SIRT1 belongs to the family of NAD+-dependent class III histone deacetylases, which regulate many physiological pathways,including a wide variety of cellular processes associated withaging,development,cell survival, and metabolism [12]. Of note, an emerging view is that SIRT1 activity may have an important impact on mitochondrial function, but the extent of the effect may be related to cell type and physiological environment [13].More importantly, a previous study showed that SIRT1 is a vital activator of peroxisome proliferator-activated receptor gamma-coactivator 1-alpha (PGC-1α) in various diseases. PGC-1α is a transcriptional coactivator that plays a central role in governing function and mitochondrial biogenesis and is known to be a positive regulator of mitochondrial function and oxidative metabolism[14, 15]. SIRT1 has been demonstrated to interact directly with PGC-1α to increase PGC-1α expression and mitochondrial biogenesis, and SIRT1-mediated promotion of mitochondrial biogenesis via PGC-1α occurs both in the nucleus and cytoplasm[16]. Previous studies have shown that activation of the SIRT1/PGC-1α pathway plays a protective role in neuronal injuries induced by the neurotoxin MPTP[17]. Additionally, in ischemic heart disease, SIRT1 suppression decreases PGC-1α[18]. In addition, a previous investigation reported that SIRT1 plays an important role in mitochondrial dysfunction in neurological diseases such as Parkinson’s disease, Alzheimer's disease, Huntington's disease, epilepsy and diabetic neuropathic pain [3].However, there have been few studies on the role of SIRT1 in mitochondrial dysfunction in CM. Considering the effects of SIRT1 on mitochondrial dysfunction, we were interested in investigating the role of SIRT1 in mitochondrial dysfunction in CM rats.

In this study,we show that the expression of SIRT1 was significantlydecreased in the TNCs of rats with CM. Moreover, SIRT1 appears to play an important role in mitochondrial dysfunction in the TNCs of CM rats.Activating SIRT1 increased the expression of PGC-1α and reversed the decreases in ATP content and mitochondrial membrane potential and alleviates the cutaneous hyperalgesia in CM rats. Therefore, SIRT1 might be a potential therapeutic target for CM.

Methods

Animals

All adult male Sprague-Dawley rats weighing 200–250 g in the study were provided by the Experimental Animal Center of Chongqing Medical University (Chongqing, China). All animals were housed in a temperature-controlled (23 ± 1°C) environment under a 12-h light/dark cycle, and all animals were allowed to access food and water freely. Before any experimental procedures were performed, all animals were acclimated for at least 7 days. The study was carried out after being approved by the Ethics Committee of the Department of Medical Research at the First Affiliated Hospital of Chongqing Medical University and all the experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996).

Surgery

The surgical procedures were performed as previously reported[19, 20]. Briefly, rats were deeply anaesthetized with 1% pentobarbital sodium (i.p., 40mg/kg body weight)and then placed on a stereotaxic

apparatus (ST-51603; Stoelting Co, Chicago, IL, USA). An incision was used to expose the skull completely, and a skull drill was used to perform 1-mm-diameter craniotomy in the right frontal bone (+ 1.5 mm from the bregma and + 1.5 mm lateral to the bregma), taking care not to damage the dura. Afterwards, a sterile stainless-steel cannula with a plastic cup was placed above the dura and affixed to the skull for delivery of IS or phosphate-buffered saline (PBS) through the cranial window. A matched obturator cap was used to seal the cannula. The skin was sutured with 4-0 nylon. After surgery, antibiotics were topically applied to prevent any infections in the operation region. Postoperative rats were placed on a thermostatic plate that was maintained at approximately 37°C until they were awake and completely mobile. The rats were housed separately and recovered for at least 7 days. After 7 days, the rats in which the surgical site was not infected and the cannula was not blocked were selected to enter the following experiment.

Infusion of inflammatory soup or PBS

The CM rat model was established by repeated infusions of IS into the dura. The rats were randomized into two groups (sham and CM) and infused with IS or PBS for 7 days as previously described [19]. The rats in the CM group were infused with 2 μl of IS, and the rats in the sham group were infused with the same volume of PBS. The inflammatory soup (IS) contained 1 mM histamine, 1 mM serotonin, 1 mM bradykinin, and 0.1 mM prostaglandin E2 in PBS (pH 7.4) (adapted from St. Louis, MO, USA). Each rat was steadily infused with 2 μl of IS or PBS for 10 min via the cannula. The tube was left in place for at least 5 min after infusion to allow the IS or PBS to diffuse into the tissue surrounding the dura, and the cap was returned to the cannula after the infusion. The rats were allowed to move freely throughout the entire procedure.

Drug administration

SRT1720 (S1129, Selleckchem), an activator of SIRT1, was intracerebroventricularly injected for three consecutive days after the 7th IS or PBS infusion. The SRT1720 was dissolved by dimethylsulphoxidein dimethylsulfoxide (DMSO). For SRT1720 administration, there were two subgroups: the CM+SRT1720 LD group (low dose, 10 μg) and the CM+SRT1720 HD group (high dose, 30 μg). The doses of SRT1720 were based on previous studies [21-23]. Intracerebroventricular injections were conducted as described previously [24]. After anesthesia, SRT1720 was injected into the lateral ventricle (-1.0 mm posterior and +1.5 mm lateral to the bregma; 4.0 mm from the skull plane) with the designated treatment solution (10 μl). An equal volume of DMSO was injected into the lateral ventricle as a corresponding vehicle control (CM+DMSO group). The rats were randomly divided into the treatment groups during the injection process.

Assessment of mechanical sensitivity and thermal hyperalgesia

The von Frey test was used to detect the mechanical threshold in the periorbital and hind paw regions. Briefly, rats were placed in a test device and adapted to the test device for 30 min before testing. The pressure threshold was determined by applying an electronic von Frey device (Electrovonfrey, model:

2450, IITC Inc., Woodland Hills, CA, USA) with assigned force values ranged from 0 to 900 g. The pressure probe tip was applied to the periorbital and hind paw regions of the rat according to the manufacturer's instructions, and the threshold was automatically recorded. The stimulation of the test was very mild at the beginning, and the stimulation increased gradually until the rats showed a positive reaction. Rapid withdrawal of the head or hind paw in response to the stimulus was considered a positive response. Each site was tested at least three times with a continuous stimulation interval of 1 min. The results for the PBS group were considered to indicate the control mechanical threshold.

Thermal hyperalgesia was classified as a significantly shortened latency of thermal stimulation of the foot. An analgesia meter (model PL-200, IITC, Taimeng, Chengdu, China) was used to test the threshold of thermal hyperalgesia. The protocol was similar to that described previously[25]. Briefly, rats were placed in a box (20*20*20 cm), and the heat source was concentrated on a portion of the hind paw of each rat. The radiant heat stimulus was delivered to the site, and the intensity of the heat source was stetted at 20%. When the hind paw moved or to prevent tissue damage after 20 seconds had passed, the stimulus ceased automatically. In all experiments, the intensity of the thermal stimulus remained consistent. The thermal stimulation was delivered to each hind paw 3 times at intervals of 5-8 mins.

To determine the effects of SRT1720 and its corresponding vehicle (DMSO) on the mechanical threshold and on thermal hyperalgesia, the same test was conducted 24 h after administration of SRT1720 or DMSO. The results are presented as the threshold in g ± the standard deviation (SD). The data were recorded separately for each time point. During the testing period, the experimenter was blinded to the groups.

Quantitative real-time polymerase chain reaction (q-PCR)

q-PCR was used to detect the mRNA expression of SIRT1 and PGC-1α. Total RNA was extracted from the TNC with RNAiso reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions, and cDNA was synthesized with a PrimeScript^{TM} RT Reagent Kit (TaKaRa). q-PCR to quantify SIRT1 and PGC-1α gene expression was performed with SYBR® Premix Ex Taq^{TM} II (TaKaRa). The gene expression is expressed as the target/reference gene expression ratio, which was determined with a post-PCR data analysis software program. The primer pairs specific for rat SIRT1, PGC-1α and GAPDH were as follows:

SIRT1(F): 5'-AAGGCCACGGATAGGTCCA -3'SIRT1(R): 5'-CCGCTTTGGTGGTTCTGAAAG-3',

PGC-1α(F): 5'-GAATGCCCGCGAACATAT-3', PGC-1α(R): 5'-CAATCCGTCTTCATCCACCG-3',

GAPDH(F): 5'-GCAAGTTCAACGGCACAG-3', and GAPDH(R): 5'-GCCAGTAGACTCCACGACAT-3'. The primers for SIRT1, PGC-1α and GAPDH were synthesized by Sangon Biotech (Shanghai, China). The reactions were incubated at 95°C for 30 s and then subjected to 45 cycles of 95°C for 5 s and 55°C for 30 s in a CFX96 Touch thermocycler (Bio-Rad, USA).

Determination of mitochondrial DNA content

Total DNA was extracted from the TNC with a mammalian genomic DNA extraction kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's instructions. q-PCR was performed with SYBR® Premix Ex Taq^{TM} II (TaKaRa). The relative mtDNA copy numbers were determined by comparing the expression of the specific mitochondrial gene ND1 to that of the GAPDH gene by the $2^{-\triangle\triangle CT}$ method. The primer pairs specific for rat ND1 and GAPDH were as follows: ND1(F): 5'-GTAATTGCGTAAGACTTAAAACC-3' , ND1(R): 5'-CCTAGAAATAAGAGGATTTAAACC-3',

GAPDH(F): 5'-GCAAGTTCAACGGCACAG-3', and GAPDH(R): 5'-GCCAGTAGACTCCACGACAT-3'. The primers for ND1 and GAPDH were synthesized by Sangon Biotech (Shanghai, China). The reactions were incubated at 95°C for 30 s and then subjected to 45 cycles of 95°C for 5 s and 55°C for 30 s in a CFX96 Touch thermocycler (Bio-Rad, USA).

Western blot analysis

Western blot analysis was used to detect the expression of SIRT1, PGC-1α, TFAM, NRF-1, NRF-2 and CGRP in the TNC (trigeminal nucleus caudalis). The left TNC was isolated immediately for further protein and gene analysis. The tissues were homogenized in RIPA lysis buffer (Beyotime, Shanghai, China) with protease inhibitor (Beyotime). The protein concentration was determined with a BCA Protein Assay Kit (Beyotime, Shanghai, China). Equal amounts of protein (40 μg) were loaded onto SDS-PA gels (Beyotime, China), electrophoresed, and transferred to PVDF membranes. Then, the membranes were blocked for 2 h at room temperature in TBST containing 5% nonfat milk and incubated at 4°C overnight with antibodies against SIRT1 (1:3000, Abcam, MA, USA), PGC-1α (1:2000, Proteintech, IL, USA), TFAM (1:1000, Novus Biologicals, Cambridge, UK), NRF-1 (1:2000, Proteintech), NRF-2 (1:2000, Proteintech), CGRP (1:3000, Abcam), and β-actin (1:5000, Proteintech). The next day, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5000, Zhongshan Golden Bridge Bio, Beijing, China) at 37 °C for 1 h. Antibody binding was visualized with a western blot detection kit (Advansta, USA) and an imaging system (Fusion, Germany). The same software was used for densitometry. β-actin was used as an internal reference to normalize the protein levels.

Immunofluorescence staining

Under deep anesthesia, rats were transcardially perfused with 0.1 M PBS and then with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The regions from medulla oblongata to the first cervical cord were separated immediately, postfixed in 4% paraformaldehyde for 24 h, and then sequentially immersed in solutions of sucrose at increasing concentrations (20% to 30%) until the tissues sank to the bottom. Segments of the TNC were cut into 10-μm-thick sections with a cryostat (Leica, Japan). The immunoreactivities of SIRT1, PGC-1α, CGRP and SP in the TNC were detected by immunofluorescence staining. First, the sections were rinsed three times with 0.1 M PBS. Following incubation with 0.3% Triton X-100 (Beyotime) for 10 min, the sections were blocked using 10% normal goat serum (Boster, Wuhan, China) at 37°C for 30 min. Then, the sections were incubated with one of the following primary antibodies at 4°C overnight: a rabbit anti-SIRT1 antibody (1:200, Bioss, China), a mouse anti-NeuN antibody (1:200,

Novus Biologicals, CO, USA), a mouse anti-PGC-1α antibody (1:200, Proteintech), a rabbit anti-NeuN antibody (1:400, Novus Biologicals, CO, USA), a mouse anti-CGRP antibody (1:50, Santa Cruz Biotechnology, CA, USA) or a mouse anti-SP antibody (1:50, Abcam). The next day, the sections were incubated with species-specific fluorophore-labeled secondary antibodies (1:400, Abbkine) for 2 h at 37°C. Then, the sections were washed in PBS three times, and the nuclei were counterstained with 4',6-diamidino-2-phenylinodole (DAPI) at 37 °C for 10 min. A confocal laser scanning fluorescence microscope (Zeiss, LSM 800, Germany) was used for visualization. The 20x objective was used to image SIRT1- and PGC-1α-immunoreactive cells. The bilateral immunoreactivity of CGRP and SP in the TNC was imaged with a 10x objective. The fluorescence intensities of CGRP and SP in the TNC were determined with ImageJ software (version 1.4.3.67, NIH) and are presented as the mean fluorescence intensities (total fluorescence intensity/area).

Determination of ATP content

Tissue ATP content was determined with an Enhanced ATP Assay Kit (Beyotime, China) following the manufacturer's instructions. The total ATP levels were calculated from the luminescence signals and were normalized by the protein concentrations.

Mitochondrial membrane potential determination

The mitochondrial membrane potential was measured with a JC-1 assay kit (Beyotime) according to the manufacturer's instructions. Fresh mitochondria were immediately isolated with a Tissue Mitochondria Isolation Kit (Beyotime) according to the manufacturer's instructions. JC-1 fluorescence was measured with a fluorescence microplate reader (M200, Tecan, Switzerland) under a single excitation wavelength (488 nm) and dual emission wavelengths (a shift from 530 to 590 nm). The changes in mitochondrial membrane potential were indicated by the ratios of the green and red fluorescence intensities.

Transmission electron microscopy

Rats were deeply anesthetized and perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 2.5% glutaraldehyde for fixation. The TNC of each rat was separated and incubated in 4% glutaraldehyde at 4°Cfor 24 h. Then, the TNC was cut into 1 mm^3 pieces. Postfixing, embedding, sectioning and staining were performed at Chongqing Medical University. Five samples were selected randomly from each rat and imaged at 50,000x magnification to assess mitochondrial ultrastructure.

Statistical analysis

All data are expressed as the mean ± SD. Statistical comparisons among groups were performed using t-tests or one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. The mechanical thresholds for the sham and CM groups were assessed by using two-way analysis of variance followed by the Bonferroni post hoc test. $P < 0.05$ was considered to indicate statistical significance in all tests.

Statistical analyses were performed in SPSS 22.0 (SPSS Inc., Chicago, IL, USA), and graphs were generated with GraphPad Prism 7 (GraphPad Software, San Diego, CA).

Results

Repeated IS infusions induced cutaneous hyperalgesia

As shown in Fig. 1, compared with those in the sham group,the mechanical thresholds of the periorbital region and the hind paw were significantly decreased in the CM group, as were the thresholds of thermal hyperalgesia. The mechanical threshold of the hind paw decreased significantly beginning at the third day (Fig. 1*A*), the threshold of the periorbital region decreased markedly beginning at the second day(Fig. 1*B*), and the threshold of thermal hyperalgesia decreased markedly on the second day(Fig. 1*C*).

IS downregulated the expression of SIRT1 in the TNC in CM rats

Western blot analysis and q-PCR were used to investigate the expression of SIRT1 protein and mRNA in the TNC in CM rats.As shown in Fig. 2, the q-PCR results showed that the mRNA level of SIRT1 was significantly lower in the CM group than in the sham group(Fig.2*A*). In addition, densitometric analysis indicated that the protein level of SIRT1 was significantly lower in the CM group than in the sham group (Fig. 2*B*). This result was consistent with the q-PCR results. Double immunofluorescence staining was used to determine the location of SIRT1 in the TNC.The results showed that SIRT1-immunoreactive cells colocalized with neurons in the TNC(Fig. 2*C*).

Activating SIRT1 alleviated IS-induced cutaneous hyperalgesia

The effect of SRT1720 treatment on mechanical allodynia was detected by using a von Frey filament, and the effect on thermal hyperalgesia was detected by using an analgesia meter. The results indicated that the cutaneous pain thresholds of the periorbital region and hind paw in the CM and vehicle (CM+DMSO) group were both significantly lower than those in the sham group. In addition, thermal hyperalgesia was decreased significantly in the CM and vehicle (CM+DMSO) group compared with the sham group. The pain thresholds of mechanical allodynia and thermal hyperalgesia in the SRT1720 LD (10 μg) and SRT1720 HD(30 μg) treatment groups were both increased significantly compared with those in the vehicle group. The pain thresholds were not significantly different between the two doses.Based on the above results, SRT1720 LD (10 μg) was chosen for the following experiments(Fig. 3*A, B, C*).

Administration of SRT1720 upregulated the expression of PGC-1α and SIRT1

To clarify the possible mechanism of SIRT1 involvement in mitochondrial dysfunction induced by repeated IS infusions, western blot analysis and q-PCR were used to detect the level of PGC-1α, a downstream regulator of SIRT1,in the TNC (Fig. 4*A, B*). The results showed that the mRNA and protein expression levels were both markedly decreased in the CM group compared with the sham group. In addition, PGC-1α expression at the mRNA or protein level was not altered in the CM+DMSO group compared with the CM group. Intracerebroventricular injections of SRT1720 significantly attenuated the

decreases in expression induced by IS infusions(Fig. 4*A, C*). To determine the location of PGC-1α in the TNC,double immunofluorescence staining was performed. Similar to SIRT1-immunoreactive cells, PGC-1α-immunoreactive cells colocalized with neurons in the TNC(Fig. 4*E*). Moreover, intracerebroventricular injections of SRT1720 significantly increased the expression of SIRT1 (Fig. 4*D*).

The level of SIRT1 regulated the mitochondria-associated proteins TFAM,NRF-1, and NRF-2

To test whether SIRT1 affects mitochondrial dysfunction in CM rats, western blot analysis was used to investigate the mitochondria-associated proteins TFAM, NRF-1, and NRF-2(Fig. 5*A*). The density analyses showed that repeated IS infusions markedly decreased the protein expression of TFAM, NRF-1 and NRF-2 in the CM group compared with the sham group. In addition, the TFAM, NRF-1 and NRF-2 protein levels were not altered in the CM+DMSO group compared with the CM group. In CM rats, intracerebroventricular injections of SRT1720 significantly increased the expression of TFAM, NRF-1 and NRF-2 compared with injections of DMSO (Fig. 5*B, C, D*).

Activating SIRT1 increased ATP and enhanced mitochondrial membrane potential

Mitochondrial ATP content and membrane potential were determined to elucidate the effects of SIRT1 on mitochondrial function. The ATP content and membrane potential were decreased dramatically in the CM group compared with the sham group(Fig. 6*A, B*). In addition,the ATP content and membrane potential were not altered in the CM+DMSO group compared with the CM group. Moreover, intracerebroventricular injections of SRT1720 significantly attenuated the decreases in ATP content and membrane potential observed in the CM+DMSO group(Fig. 6*A, B*).

The level of SIRT1 regulated the expression of mtDNA

To test whether SIRT1 affects mitochondrial dysfunction in CM rats, q-PCR was used to investigate mtDNA. The results showed that repeated IS infusions significantly decreased the expression of mtDNA in the CM group compared with the sham group. The expression of mtDNA was not altered in the CM+DMSO group compared with the CM group. Intracerebroventricular injections of SRT1720 significantly attenuated the decrease in mtDNA observed in the CM+DMSO group(Fig. 6*C*).

Activating SIRT1 suppressed mitochondrial morphological abnormalities in CM rats

Transmission electron microscopy was used to assess the mitochondrial ultrastructure in TNC neurons. The results showed that there was significant mitochondrial swelling in the CM group, and the number of mitochondrial cristae was significantly reduced in the CM group compared with the sham group. Additionally, vacuoles appeared in the mitochondria of the CM group. However, in CM rats, SRT1720 treatment reduced the swelling of the mitochondria, reduced the appearance of vacuoles, and increased the number of cristae compared with DMSO treatment(Fig. 6*D*).

Activating SIRT1 decreased the expression of CGRP and SP in the TNC

To investigate the contribution of SIRT1 activation to the alleviation of CM, we measured the expression of calcitonin gene-related peptide(CGRP) and substance P(SP) in the TNC(Fig. 7*A, B*).CGRP and SP are important in migraine pathophysiology. Western blot analysis and immunofluorescence staining were used to investigate the expression of CGRP, and immunofluorescence staining was used to test the expression of SP in the TNC. Western blot analysis showed that the level of CGRP was increased markedly in the CM group compared with the sham group. There were no significant differences between the CM group and the CM+DMSO group. Moreover, intracerebroventricular injections of SRT1720 significantly reduced the levels of CGRP(Fig. 7*A*). Furthermore, the fluorescence intensity of CGRP in the TNC was higher in the CM group than in the sham group. SRT1720 treatment weakened the elevation in CGRP immunoreactivity induced by IS infusions, which was consistent with the western blotting results(Fig. 7*C*). The same result was also obtained for SP(Fig. 7*D*).

Discussion

Abnormal mitochondrial function and morphology may cause various diseases. Mitochondrial integrity is vital for organ function and cells because of its role in ATP generation, lipid and fatty acid metabolism, signaling pathways and programmed cell death. In addition, mitochondrial dysfunction may play a role in CM. However, there are no effective treatments targeting mitochondrial dysfunction for CM.

The present study is the first to investigate alterations in SIRT1 in the TNCs of CM rats and to investigate the role of SIRT1 in mitochondrial dysfunction in the TNCs of CM rats. First, in our study, the pain thresholds of mechanical allodynia and thermal hyperalgesia in CM rats were found to be significantly decreased, which indicated that the model was established successfully. Second, we found that the expression of SIRT1 was decreased in the TNC in the CM group and that SIRT1 was expressed in TNC neurons. Further experiments showed that activation of SIRT1 by SRT1720 alleviated mitochondrial dysfunction and cutaneous hyperalgesia in CM rats. Moreover, our results demonstrated that SIRT1 plays a significant role in mediating the expression of PGC-1α. Activation of SIRT1 reversed the decrease in PGC-1α in CM rats. Notably, activating SIRT1 reversed the reductions in TFAM, NRF-1, and NRF-2, which are mitochondria-associated proteins, in CM rats, and it reversed the decline in mtDNA in CM rats as well. Moreover, SIRT1 activation increased mitochondrial ATP content and mitochondrial membrane potential in CM rats. In summary, SIRT1 plays a regulatory role in PGC-1α expression in the TNC, and SIRT1 plays an important role in reversing mitochondrial dysfunction in CM.

SIRT1, a class III histone deacetylase, is an important member of the sirtuin family. It exerts beneficial effects in cancer, diabetes, cardiovascular disease and aging by regulating various transcription factors, such as ether-a-go-go-related gene 1 (Erg-1), forkhead box O-type protein (FOXO), p65, p53 and PGC-1α[26, 27]. Here, our study found that the expression of SIRT1 was decreased both at the mRNA and protein levels in CM rats.Similar results have been reported in the diabetic neuropathic pain. In addition,

our results also showed SIRT1 expression in neurons of the TNC. Our study results suggested that activation of SIRT1 with SRT1720 significantly increased the threshold of pain and increased recovery of mitochondrial function after CM. Similar results have been reported after intracerebral hemorrhage (ICH) [28]. These findings suggest that SIRT1 plays a crucial role in CM and may be a potential therapeutic target for CM.

A previous study showed that SIRT1 is a vital activator of PGC-1α in various diseases and that there are two pathways for SIRT1-mediated regulation of PGC-1α: control of PGC-1α activity and regulation of PGC-1α [29]. As one of the important downstream targets of SIRT1, PGC-1α controls mitochondrial capacity in neurons, and neuronal PGC-1α could be activated by SIRT1 deacetylase[30]. Moreover, increasing PGC-1α or activity can reverse the phenotypic consequences of mitochondrial damage, and overexpression of PGC-1α can fully reverse mitochondrial loss [30]. In addition, PGC-1α overexpression protects neurons against neurodegenerative insults[17, 31]. These findings imply that PGC-1α contributes to the protection of neurons. In the present study, themRNA and protein levels of PGC-1α were decreased in rats subjected to CM. In contrast, SIRT1 activation prevented this reduction and increased PGC-1α mRNA and protein expression. Considering that PGC-1αincreased after administration of SRT1720 and that PGC-1α plays a central role in governing mitochondrial capacity, we speculate that SIRT1 might reverse mitochondrial dysfunction and protect mitochondrial function in CM by increasing the expression of PGC-1α.

Furthermore, to examine whether SIRT1 protects mitochondrial function in rats after CM, we evaluated ATP content and mitochondrial membrane potential and assessed mitochondria-associated proteins,mtDNA and mitochondrial structure. TFAM, NRF-1, and NRF-2 are downstream proteins of PGC-1α that are also mitochondria-associated proteins. Mitochondrial biogenesis is tightly regulated by PGC-1α and its downstream proteins TFAM, NRF-1 and NRF-2[32]. Mitochondrial biogenesis can increase mitochondrial numbers to accommodate energy demands in response to different physiologic and pathologic conditions [33]. TFAM is a key regulator of mtDNA replication and transcription[34]. NRF-1 stimulates the transcription of TFAM by binding to an NRF-1 response element in its promoters. PGC-1α stimulates the expression of NRF-1 and coactivates the transcription function of NRF-1 on the promoter of TFAM. NRF-2 plays a crucial role in maintaining mitochondrial structural and functional integrity under conditions of oxidative and inflammatory stress[35]. In addition, overexpression of TFAM and NRF-1 protects against mitochondrial dysfunction in neuronal cells[36].In our study, mitochondrial proteins such as TFAM, NRF-1, and NRF-2 were reduced in the TNC in the CM group compared with the sham group, and similar findings have also been reported in trigeminal ganglion neurons in another rat model of migraine[11]. Consistent with our hypothesis, the results showed that activation of SIRT1 by SRT1720 could mostly restore mitochondrial proteins and increase the expression of SIRT1 in the TNC in CM rats.Similar results were reported in mouse podocytes cultured under high-glucose conditions[37]. In short, our results indicated that activating SIRT1 and thus increasing the expression of PGC-1α protects against mitochondrial dysfunction by increasing the levels of mitochondrial proteins such as TFAM, NRF-1, and NRF-2.The previous study shown that the levels of key mitochondrial electron transport chain proteins changes was caused by the changes of mtDNA integrity and number in neurons and the changes of mtDNA integrity and number in neurons caused energy production defects which may

represent an underlying mechanism for migraine pathogenesis[8, 38]. In 1988, the deficit in mitochondrial morphology was found in migraine sufferers [8].Furthermore, our results showed that mitochondrial DNA copy number was increased, mitochondrial structural changes were attenuated, and SIRT1 expression was increased in the CM + SRT1720 group compared with the CM + DMSO group.The results indicated that activation of SIRT1 attenuated mitochondrial dysfunction by increasing mtDNA content and attenuating mitochondrial structural changes. ATP and adenosine play an important role in the nervous system[39]. And the mitochondrial membrane potential is crucial for energy metabolism in the mitochondria. Of note, Reyngoudt et al, who found a significant decrease compared with controls about the contents of ATP in the occipital lobe of migraine patients [40]. And significantly increased ADP was found in the migraine patients, indicating that brain tissue has a lower energy reserve and is working at a higher metabolic rate [41, 42]. What's more, the decline of ATP content and mitochondrial membrane potential were found in the nitroglycerin rat model of migraine[43]. In this study, we also verified that increases in mitochondrial elements correlated with improved mitochondrial function by assessing ATP content and mitochondrial membrane potential after activating SIRT1. Consistent with previous studies, the results showed that the ATP content and mitochondrial membrane potential were decreased markedly in the CM group compared with sham group,and the ATP content and mitochondrial membrane potential were increased significantly in the CM + SRT1720 group compared with CM + DMSO group. These findings are consistent with the results of a previous study in which activation of SIRT1 and PGC-1α by resveratrol was found to improve mitochondrial function [44].Thus, these results indicate that SIRT1 exerts an important protective effect against mitochondrial dysfunction in CM.

CGRP and SP are important in migraine pathophysiology, are expressed in trigeminal ganglia and are involved in trigeminovascular innervation and modulation of nociceptive transmission. A previous study showed that during a migraine attack,the levels of CGRP and SP in plasma are increased [45, 46].Additionally, these proteins are used as biological markers of neuronal activation and central sensitization [46, 47]. Of note, CGRP could act as a biomarker of migraine[48]. Thus, we used CGRP and SP to evaluate the effects of SIRT1 activation on CM rats. In our study, upregulated expression of CGRP and SP was observed in the TNC in CM rats. This finding is in line with the results of a previous study [47]. In addition, activation of SIRT1 downregulated the levels of CGRP and SP in the TNC, which indicates that activating SIRT1 to restore mitochondrial dysfunction might alleviate CM. Notably, SIRT1 activation might play a positive role in alleviating neuronal activation and central sensitization by alleviating mitochondrial dysfunction in the context of CM, because CGRP and SP are known biological markers of neuronal activation and central sensitization.

The present study showed that SIRT1 might play a therapeutic role in mitochondrial dysfunction in CM rats and that the effect of SIRT1 on mitochondrial dysfunction might be mediated by activation of the expression of PGC-1α. However, our study has some limitations.First, we only tested the effect of SIRT1 on mitochondrial dysfunction in vivo; its effect should be explored in cultured neurons and other types of cells as well.Second, based on our study,we speculate that the SIRT1/PGC-1α pathway plays an important role in alleviating mitochondrial dysfunction, but the specific mechanism of SIRT1/PGC-1αin mitochondrial dysfunction in CM requires further exploration.In addition, the relationship between SIRT1

and central sensitization requires further investigation. Therefore, in the future, we will conduct more investigations to explore the specific function and mechanism of SIRT1/PGC-1α in mitochondrial dysfunction in the context of CM and to explore the relationship between SIRT1 and central sensitization.

Conclusion

This is the first study to explore the changes of SIRT1 in CM and to investigate the role of SIRT1 in CM.In conclusion, our results demonstrate that SIRT1was decreased markedly in TNC in CM and activation of SIRT1 contributes to alleviation of CM by ameliorating mitochondrial dysfunction. Collectively, our data suggest that activation of SIRT1 may be a target for new therapies to prevent mitochondrial dysfunction in patients with CM.

Abbreviations

CM, chronic migraine; CGRP, calcitonin gene-related peptide; DMSO, dimethylsulphoxidein dimethlsulfoxide;EM, episodic migraine; IS, inflammatory soup; mtDNA, mitochondrial DNA; NRF-1, nuclear respiratory factor 1; NRF-2, nuclear respiratory factor 2; PBS, phosphate-buffered saline; PGC-1α, peroxisome proliferator-activated receptor gamma-coactivator 1-alpha; SIRT1, Silent information regulator 1; SP, substance P; TFAM, transcription factor A; TNC, trigeminal nucleus caudalis.

Declarations

Acknowledgements

We thank all those who participated in this study.

Funding

This work was supported by the National Natural Science Foundation of China (No: 81671093), the District Science and Technology Projects of Yuzhong Chongqing (No: 20160107) and the Natural Science Foundation Project of Chongqing(No:cstc2017jcyjBX0043).

Availability of data and materials

Data can be made available upon request.

Author Contributions

Jie Liang conceived and designed the study, collected and analysed the data, and drafted the manuscript.Li-Xue Chen wrote the proposal, conceived of the study, supervised the experiment and critically revised the manuscript. Ji-Ying Zhou designed the study and critically revised the manuscript. Xue Zhou, Jiang Wang and Zhao-Yang Fei collected the data and critically revised the manuscript.

Guang-Cheng Qin and Dun-Ke Zhang performed the data analysis and reviewed the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee for Animal Experimentation of Chongqing Medical University (Document number SYXK (□) 2012–0001) and the data presented in our manuscript were collected from animals.

Consent for publication

Not applicable.

Conflict of Interest

The authors declare that they have no competing interests.

References

1. Dodick DW: **Migraine.** *Lancet* 2018, **391:**1315-1330.
2. Group GBDNDC: **Global, regional, and national burden of neurological disorders during 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015.** *Lancet Neurol* 2017, **16:**877-897.
3. Headache Classification Committee of the International Headache S: **The International Classification of Headache Disorders, 3rd edition (beta version).** *Cephalalgia* 2013, **33:**629-808.
4. Schwedt TJ: **Chronic migraine.** *BMJ* 2014, **348:**g1416.
5. Lipton RB, Fanning KM, Serrano D, Reed ML, Cady R, Buse DC: **Ineffective acute treatment of episodic migraine is associated with new-onset chronic migraine.** *Neurology* 2015, **84:**688-695.
6. Islam MT: **Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders.** *Neurol Res* 2017, **39:**73-82.
7. Lin MT, Beal MF: **Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases.** *Nature* 2006, **443:**787-795.
8. Yorns WR, Jr., Hardison HH: **Mitochondrial dysfunction in migraine.** *Semin Pediatr Neurol* 2013, **20:**188-193.
9. Stuart S, Griffiths LR: **A possible role for mitochondrial dysfunction in migraine.** *Mol Genet Genomics* 2012, **287:**837-844.
10. Colombo B, Saraceno L, Comi G: **Riboflavin and migraine: the bridge over troubled mitochondria.** *Neurol Sci* 2014, **35 Suppl 1:**141-144.
11. Dong X, Guan X, Chen K, Jin S, Wang C, Yan L, Shi Z, Zhang X, Chen L, Wan Q: **Abnormal mitochondrial dynamics and impaired mitochondrial biogenesis in trigeminal ganglion neurons in a rat model of migraine.** *Neurosci Lett* 2017, **636:**127-133.

12. Houtkooper RH, Pirinen E, Auwerx J: **Sirtuins as regulators of metabolism and healthspan.** *Nat Rev Mol Cell Biol* 2012, **13:**225-238.
13. Tang BL: **Sirt1 and the Mitochondria.** *Mol Cells* 2016, **39:**87-95.
14. Wang SJ, Zhao XH, Chen W, Bo N, Wang XJ, Chi ZF, Wu W: **Sirtuin 1 activation enhances the PGC-1alpha/mitochondrial antioxidant system pathway in status epilepticus.** *Mol Med Rep* 2015, **11:**521-526.
15. Fernandez-Marcos PJ, Auwerx J: **Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis.** *Am J Clin Nutr* 2011, **93:**884S-890.
16. Brenmoehl J, Hoeflich A: **Dual control of mitochondrial biogenesis by sirtuin 1 and sirtuin 3.** *Mitochondrion* 2013, **13:**755-761.
17. Mudo G, Makela J, Di Liberto V, Tselykh TV, Olivieri M, Piepponen P, Eriksson O, Malkia A, Bonomo A, Kairisalo M, et al: **Transgenic expression and activation of PGC-1alpha protect dopaminergic neurons in the MPTP mouse model of Parkinson's disease.** *Cell Mol Life Sci* 2012, **69:**1153-1165.
18. Hu MZ, Zhou B, Mao HY, Sheng Q, Du B, Chen JL, Pang QF, Ji Y: **Exogenous Hydrogen Sul fi de Postconditioning Protects Isolated Rat Hearts From Ischemia/Reperfusion Injury Through Sirt1/PGC-1alpha Signaling Pathway.** *Int Heart J* 2016, **57:**477-482.
19. Melo-Carrillo A, Lopez-Avila A: **A chronic animal model of migraine, induced by repeated meningeal nociception, characterized by a behavioral and pharmacological approach.** *Cephalalgia* 2013, **33:**1096-1105.
20. Oshinsky ML, Gomonchareonsiri S: **Episodic dural stimulation in awake rats: a model for recurrent headache.** *Headache* 2007, **47:**1026-1036.
21. Gueguen C, Palmier B, Plotkine M, Marchand-Leroux C, Besson VC: **Neurological and histological consequences induced by in vivo cerebral oxidative stress: evidence for beneficial effects of SRT1720, a sirtuin 1 activator, and sirtuin 1-mediated neuroprotective effects of poly(ADP-ribose) polymerase inhibition.** *PLoS One* 2014, **9:**e87367.
22. Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB, et al: **Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes.** *Nature* 2007, **450:**712-716.
23. Smith JJ, Kenney RD, Gagne DJ, Frushour BP, Ladd W, Galonek HL, Israelian K, Song J, Razvadauskaite G, Lynch AV, et al: **Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction in vivo.** *BMC Syst Biol* 2009, **3:**31.
24. Wu B, Wang S, Qin G, Xie J, Tan G, Zhou J, Chen L: **Protein Kinase C gamma Contributes to Central Sensitization in a Rat Model of Chronic Migraine.** *J Mol Neurosci* 2017, **63:**131-141.
25. Liu S, Liu YP, Song WB, Song XJ: **EphrinB-EphB receptor signaling contributes to bone cancer pain via Toll-like receptor and proinflammatory cytokines in rat spinal cord.** *Pain* 2013, **154:**2823-2835.
26. Baur JA: **Biochemical effects of SIRT1 activators.** *Biochim Biophys Acta* 2010, **1804:**1626-1634.
27. Herranz D, Serrano M: **SIRT1: recent lessons from mouse models.** *Nat Rev Cancer* 2010, **10:**819-823.

28. Zhou Y, Wang S, Li Y, Yu S, Zhao Y: **SIRT1/PGC-1alpha Signaling Promotes Mitochondrial Functional Recovery and Reduces Apoptosis after Intracerebral Hemorrhage in Rats.** *Front Mol Neurosci* 2017, **10:**443.
29. Tsuruoka S, Hiwatashi A, Usui J, Yamagata K: **The mitochondrial SIRT1-PGC-1alpha axis in podocyte injury.** *Kidney Int* 2012, **82:**735-736.
30. Wareski P, Vaarmann A, Choubey V, Safiulina D, Liiv J, Kuum M, Kaasik A: **PGC-1{alpha} and PGC-1{beta} regulate mitochondrial density in neurons.** *J Biol Chem* 2009, **284:**21379-21385.
31. Shin JH, Ko HS, Kang H, Lee Y, Lee YI, Pletinkova O, Troconso JC, Dawson VL, Dawson TM: **PARIS (ZNF746) repression of PGC-1alpha contributes to neurodegeneration in Parkinson's disease.** *Cell* 2011, **144:**689-702.
32. Scarpulla RC, Vega RB, Kelly DP: **Transcriptional integration of mitochondrial biogenesis.** *Trends Endocrinol Metab* 2012, **23:**459-466.
33. Ploumi C, Daskalaki I, Tavernarakis N: **Mitochondrial biogenesis and clearance: a balancing act.** *FEBS J* 2017, **284:**183-195.
34. Campbell CT, Kolesar JE, Kaufman BA: **Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number.** *Biochim Biophys Acta* 2012, **1819:**921-929.
35. Dinkova-Kostova AT, Abramov AY: **The emerging role of Nrf2 in mitochondrial function.** *Free Radic Biol Med* 2015, **88:**179-188.
36. Piao Y, Kim HG, Oh MS, Pak YK: **Overexpression of TFAM, NRF-1 and myr-AKT protects the MPP(+)-induced mitochondrial dysfunctions in neuronal cells.** *Biochim Biophys Acta* 2012, **1820:**577-585.
37. Zhang T, Chi Y, Ren Y, Du C, Shi Y, Li Y: **Resveratrol Reduces Oxidative Stress and Apoptosis in Podocytes via Sir2-Related Enzymes, Sirtuins1 (SIRT1)/Peroxisome Proliferator-Activated Receptor gamma Co-Activator 1alpha (PGC-1alpha) Axis.** *Med Sci Monit* 2019, **25:**1220-1231.
38. Carelli V, Chan DC: **Mitochondrial DNA: impacting central and peripheral nervous systems.** *Neuron* 2014, **84:**1126-1142.
39. Chen KH, Lin CR, Cheng JT, Cheng JK, Liao WT, Yang CH: **Altered mitochondrial ATP synthase expression in the rat dorsal root ganglion after sciatic nerve injury and analgesic effects of intrathecal ATP.** *Cell Mol Neurobiol* 2014, **34:**51-59.
40. Reyngoudt H, Paemeleire K, Descamps B, De Deene Y, Achten E: **31P-MRS demonstrates a reduction in high-energy phosphates in the occipital lobe of migraine without aura patients.** *Cephalalgia* 2011, **31:**1243-1253.
41. Kemp GJ: **Non-invasive methods for studying brain energy metabolism: what they show and what it means.** *Dev Neurosci* 2000, **22:**418-428.
42. Reyngoudt H, Achten E, Paemeleire K: **Magnetic resonance spectroscopy in migraine: what have we learned so far?** *Cephalalgia* 2012, **32:**845-859.

43. Li R, Liu Y, Chen N, Zhang Y, Song G, Zhang Z: **Valproate Attenuates Nitroglycerin-Induced Trigeminovascular Activation by Preserving Mitochondrial Function in a Rat Model of Migraine.** *Med Sci Monit* 2016, **22:**3229-3237.
44. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, et al: **Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha.** *Cell* 2006, **127:**1109-1122.
45. Fusayasu E, Kowa H, Takeshima T, Nakaso K, Nakashima K: **Increased plasma substance P and CGRP levels, and high ACE activity in migraineurs during headache-free periods.** *Pain* 2007, **128:**209-214.
46. Tajti J, Szok D, Majlath Z, Tuka B, Csati A, Vecsei L: **Migraine and neuropeptides.** *Neuropeptides* 2015, **52:**19-30.
47. Iyengar S, Ossipov MH, Johnson KW: **The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine.** *Pain* 2017, **158:**543-559.
48. Durham P, Papapetropoulos S: **Biomarkers associated with migraine and their potential role in migraine management.** *Headache* 2013, **53:**1262-1277.

Figures

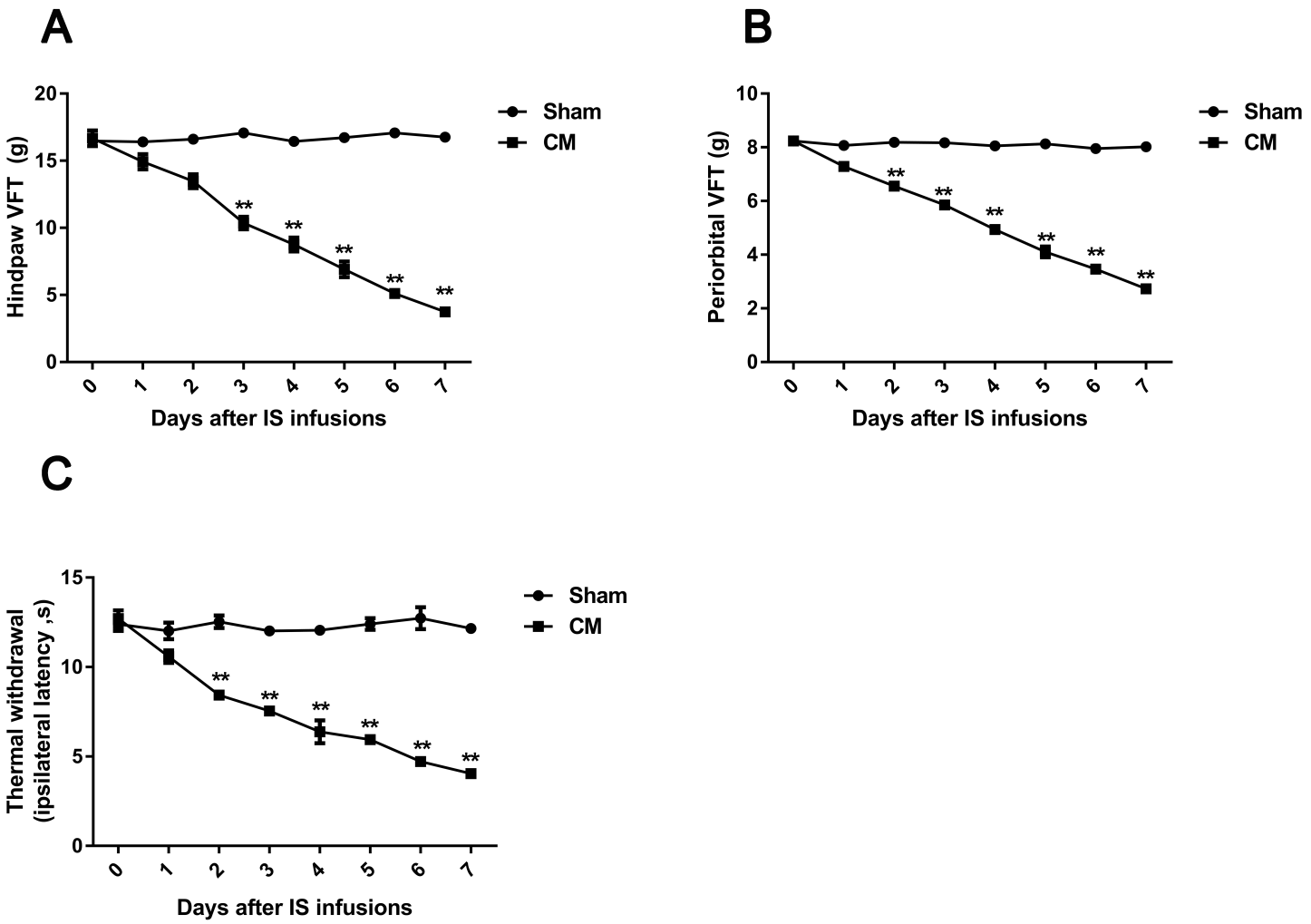

Figure 1

Development of cutaneous hyperalgesia in rats. A)In the CM group, the threshold of the hind paw decreased significantly beginning on the third day compared with that in the sham group. B) In the CM group, the threshold of the periorbital region decreased markedly beginning on the second day. C) In the CM group,the threshold of thermal hyperalgesia decreased markedly on the second day. (n = 10 per group, **P < 0.01)

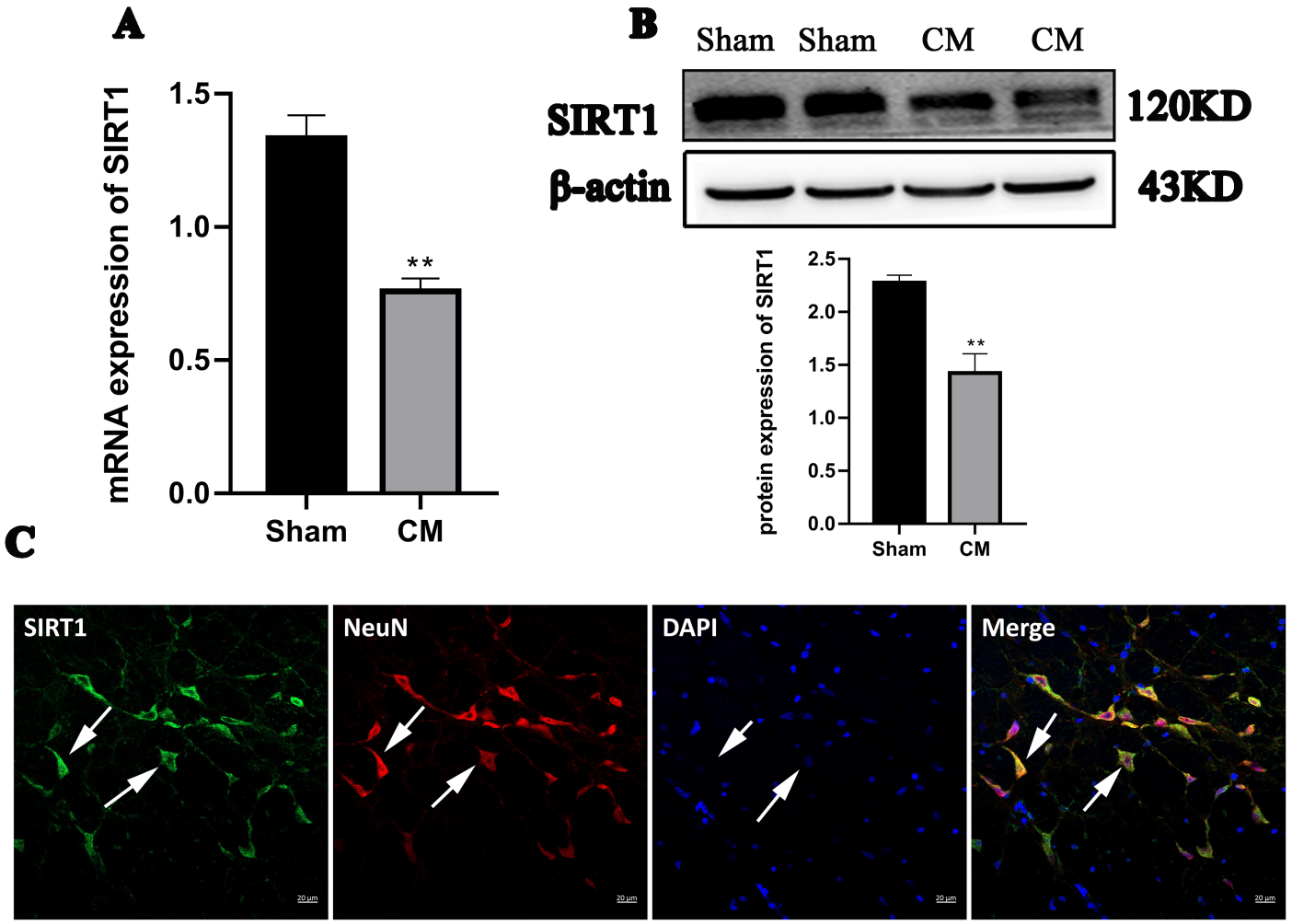

Figure 2

Expression and location of SIRT1 in the TNC in CM rats. A)The mRNA level of SIRT1 in the TNC in the CM group was decreased markedly compared with that in the sham group(n=6 per group, **P<0.01 compared with the sham group). B) Representative western blots of SIRT1. The expression of SIRT1 protein in the TNC in the CM group was decreased significantly compared with that in the sham group(n=6 per group, **P<0.01 compared with the sham group). C)The location of SIRT1. SIRT1-immunoreactive cells colocalized with neurons in the TNC(n=6 per group, scale bar=20 μm).

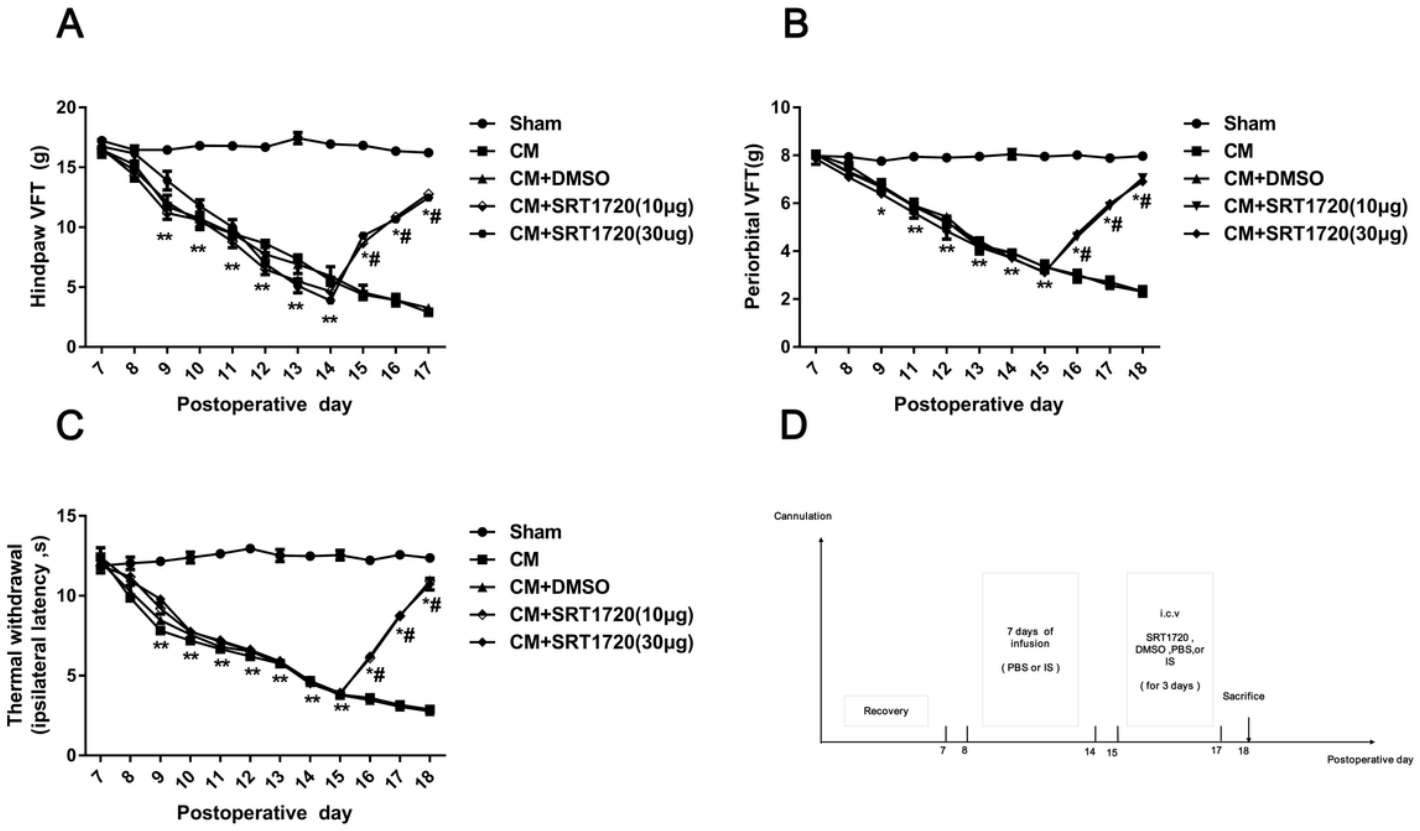

Figure 3

The thresholds of mechanical allodynia and thermal hyperalgesia after administration of SRT1720. On the postoperative 15,16 and 17 days, the sham group dural infused PBS, the CM group dural infused IS, the CM+SRT1720 groups intracerebroventricularly injected SRT1720, the CM+DMSO group intracerebroventricularly injected DMSO. A)Compared with the sham group, the CM group showed significant decreases in the paw withdrawal thresholds, but there was no significant difference in the von Frey test results between the CM and CM + DMSO groups. Compared with DMSO administration, SRT1720 administration significantly increased the periorbital pressure thresholds in CM rats. There was no significant difference in the von Frey test results between the CM +SRT1720 LD (10 μg) and CM +SRT1720 HD (30 μg) groups. B)Compared with the sham group, the CM group showed significant decreases in the periorbital pressure thresholds, but no significant difference in the von Frey test was found between the CM and CM + DMSO groups. Compared withDMSO administration, SRT1720 administration significantly increased the periorbital pressure thresholds in CM rats. There was no significant difference in the von Frey test results between the CM +SRT1720 LD (10 μg) and CM +SRT1720 HD (30 μg) groups. C)Compared with the sham group, the CM group showed significant decreases in the thermal hyperalgesia thresholds, but there was no significant difference between the CM and CM + DMSO groups. Compared withDMSO administration, SRT1720 administration significantly increased the thermal hyperalgesia thresholds. There was no significant difference between the CM +SRT1720 LD (10 μg) and CM +SRT1720 HD (30 μg) groups(n = 10 per group, *P< 0.01 compared with

the sham group, *#P< 0.01 compared with the CM + DMSO group and sham group). D)The process of the experiment.

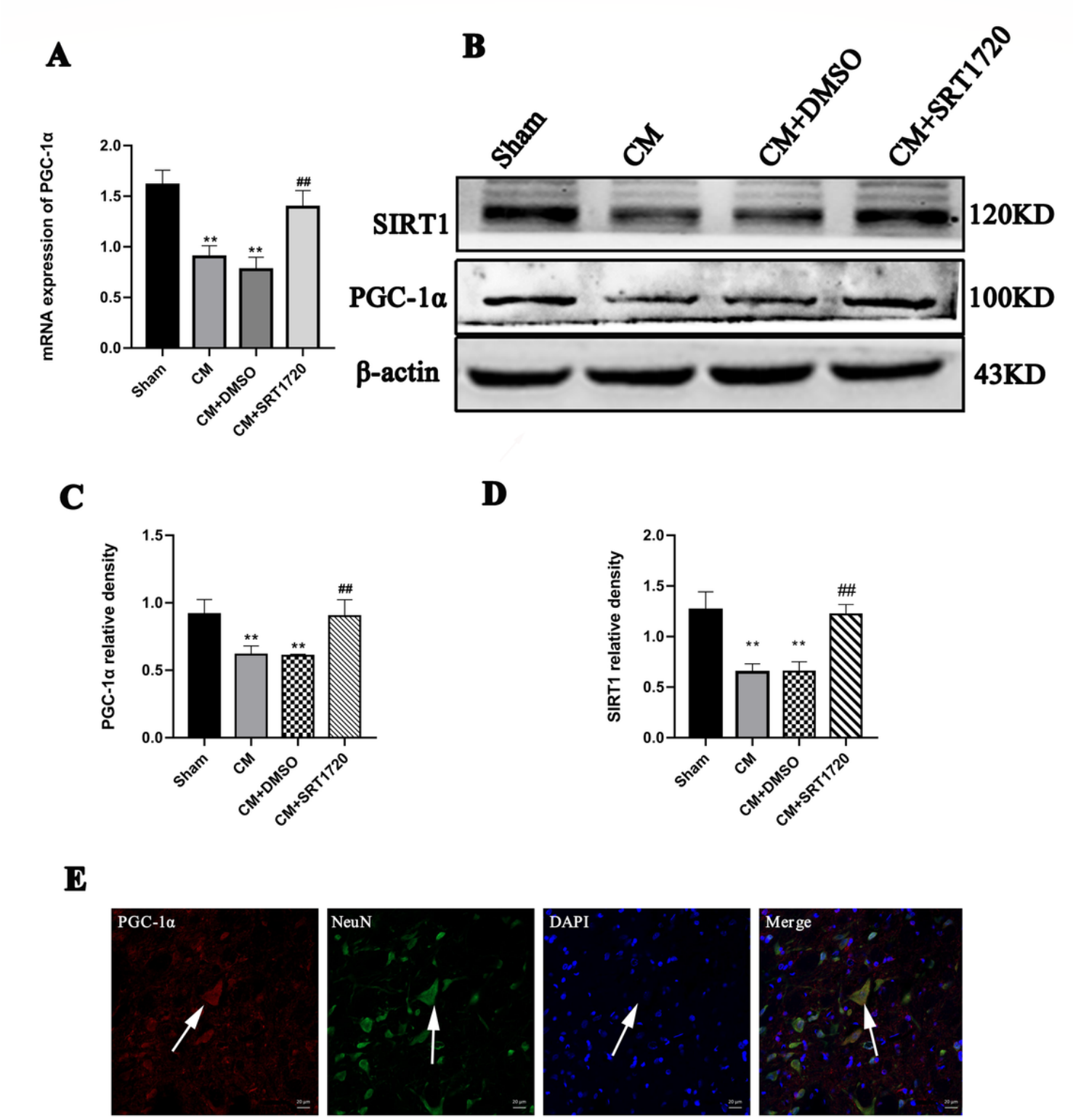

Figure 4

The expression of PGC-1α and SIRT1 in each group and the location of PGC-1α in the TNC. A) The mRNA expression of PGC-1α was decreased significantly in the CM group compared with the sham group. Compared with that in the CM+DMSO group, the mRNA level of PGC-1α was increased markedly in the

CM+SRT1720 group(n=6 per group, **P<0.01 compared with the sham group, ## P<0.01 compared with the CM+DMSO group). B)The three rows show SIRT1, PGC-1α and β-actin. C)The protein level of PGC-1α was lower in the CM group than in the sham group. Compared with that in the CM+DMSO group, the level ofPGC-1α protein was increased significantly in the CM+SRT1720 group(n=6 per group, **P<0.01 compared with the sham group, ## P<0.01 compared with the CM+DMSO group). D) The protein level of SIRT1 was lower in the CM group than in the sham group. Compared with that in the CM+DMSO group, the protein level of SIRT1 was increased markedly in the CM+SRT1720 group (n=6 per group, **P<0.01 compared with the sham group, ## P<0.01 compared with the CM+DMSO group). E) The location of PGC-1α in the TNC. PGC-1α-immunoreactive cells colocalized with neurons in the TNC (n=6 per group, scale bar=20 μm).

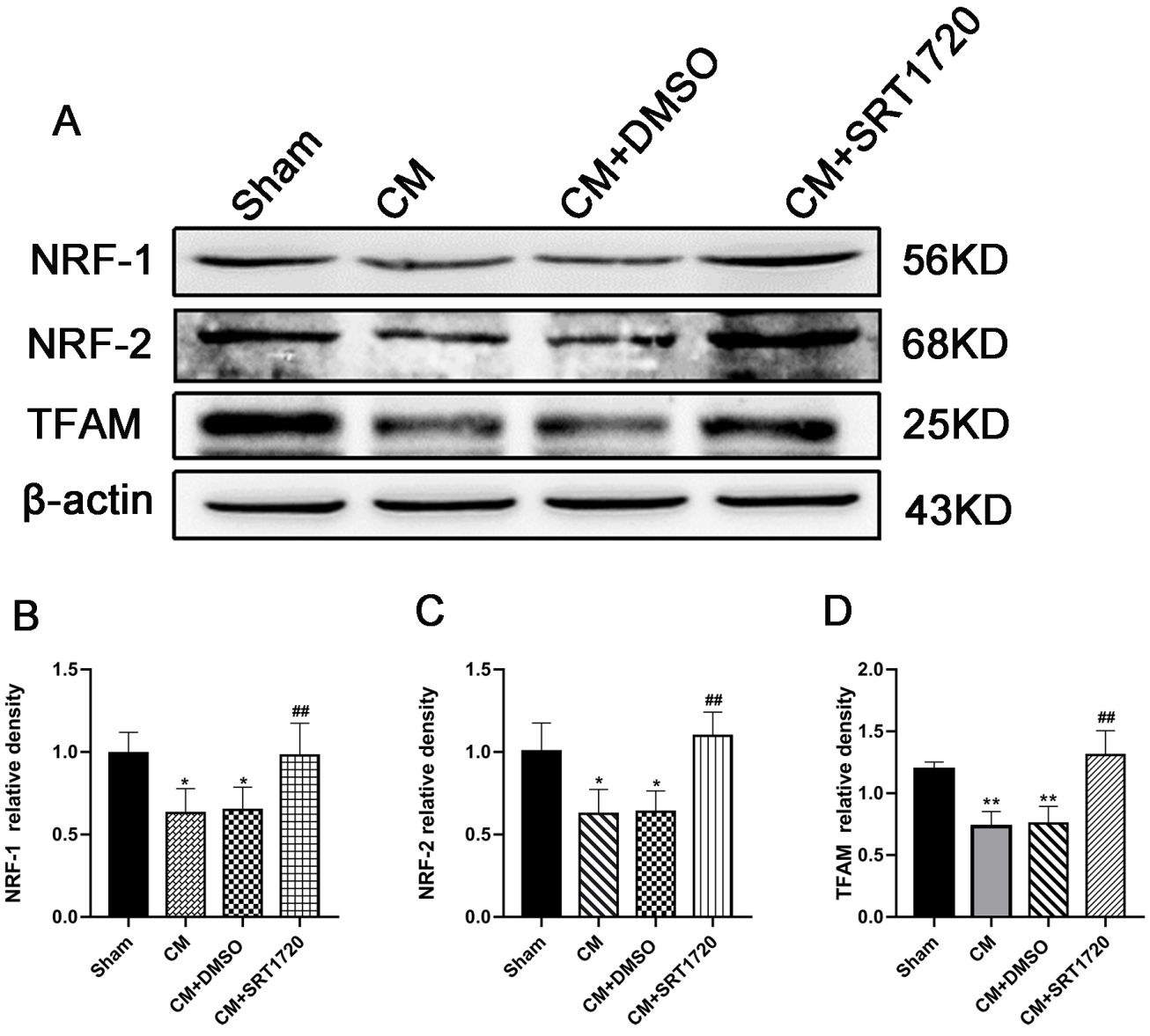

Figure 5

The expression of TFAM, NRF-1, and NRF-2 in the TNC in each group. A) The four rows show NRF-1, NRF-2, TFAM and β-actin. B) The protein levels of NRF-1 in the TNC were lower in the CM group than in the sham group. In CM rats, compared with DMSO, SRT1720 significantly increased the levels of NRF-1. C)

The protein levels of NRF-2 in the TNC were lower in the CM group than in the sham group. In CM rats, compared with DMSO, SRT1720 significantly increased the level of NRF-2. D) The protein levels of TFAM in the TNC were lower in the CM group than in the sham group. In CM rats, compared with DMSO, SRT1720 significantly increased the level of TFAM(n = 6 in each group, *P<0.05 compared with the sham group,**P < 0.01 compared with the sham group, ##P< 0.01 compared with the CM + DMSO group).

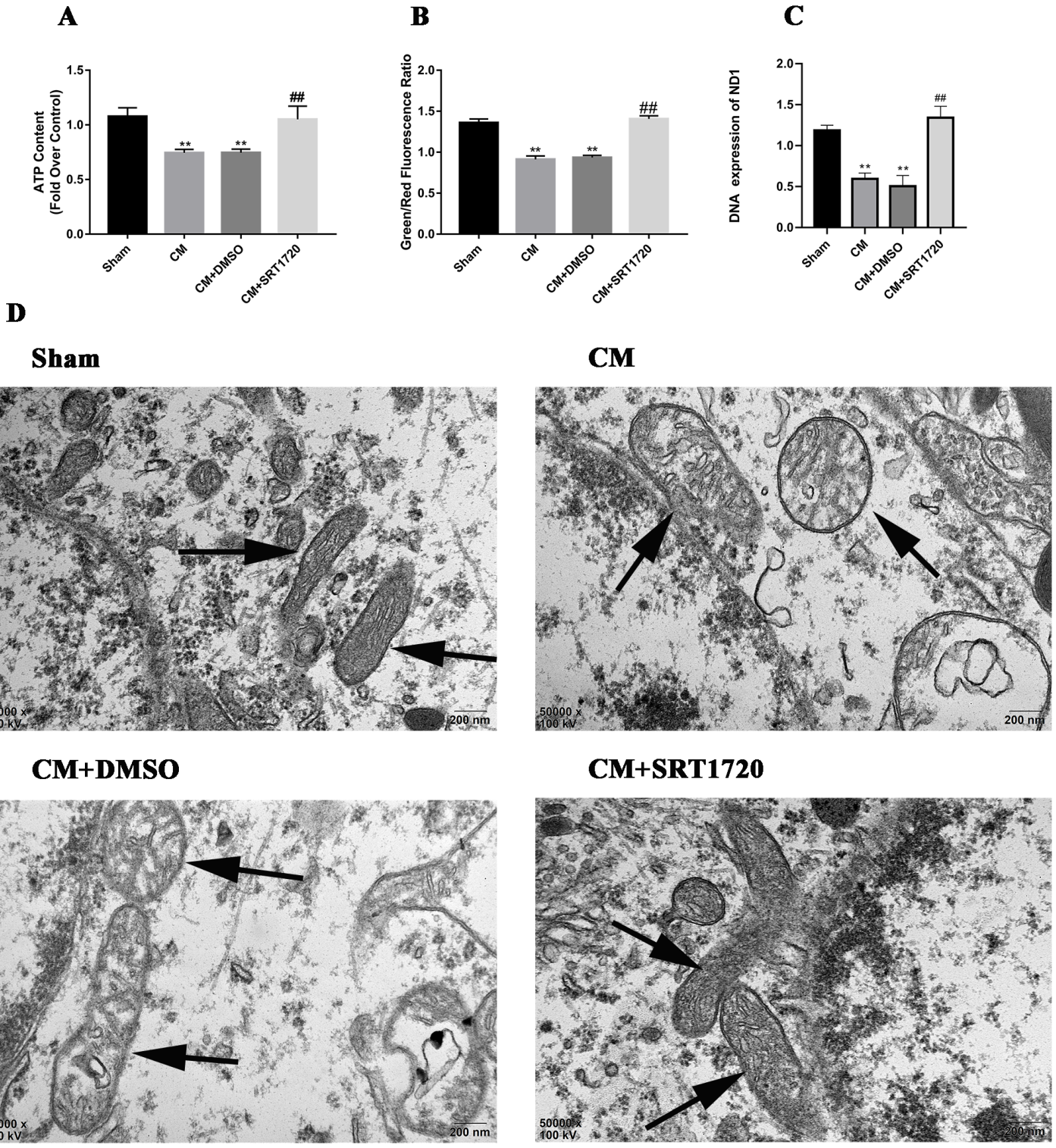

Figure 6

Changes in ATP content, mitochondrial membrane potential, mtDNA, and mitochondrial morphology in each group. A)The ATP content was decreased dramatically in the CM group compared with the sham group, and intracerebroventricular injections of SRT1720 significantly attenuated the decrease in ATP content observed in the CM+DMSO group. B) The mitochondrial membrane potential was decreased dramatically in the CM group compared with the sham group, and intracerebroventricular injections of SRT1720 significantly attenuated the decrease in mitochondrial membrane potential observed in the CM+DMSO group. C) The expression of ND1 was decreased dramatically in the CM group compared with the sham group, and intracerebroventricular injections of SRT1720 significantly attenuated the decrease in the expression of ND1 observed in the CM+DMSO group. D)Abnormal mitochondrial morphology was found in the CM group, but intracerebroventricular injections of SRT1720 reduced the abnormality of mitochondrial structure. (n = 6 in each group, **P< 0.01 compared with the sham group, ##P< 0.01 compared with the CM + DMSO group, scale bars = 200 nm)

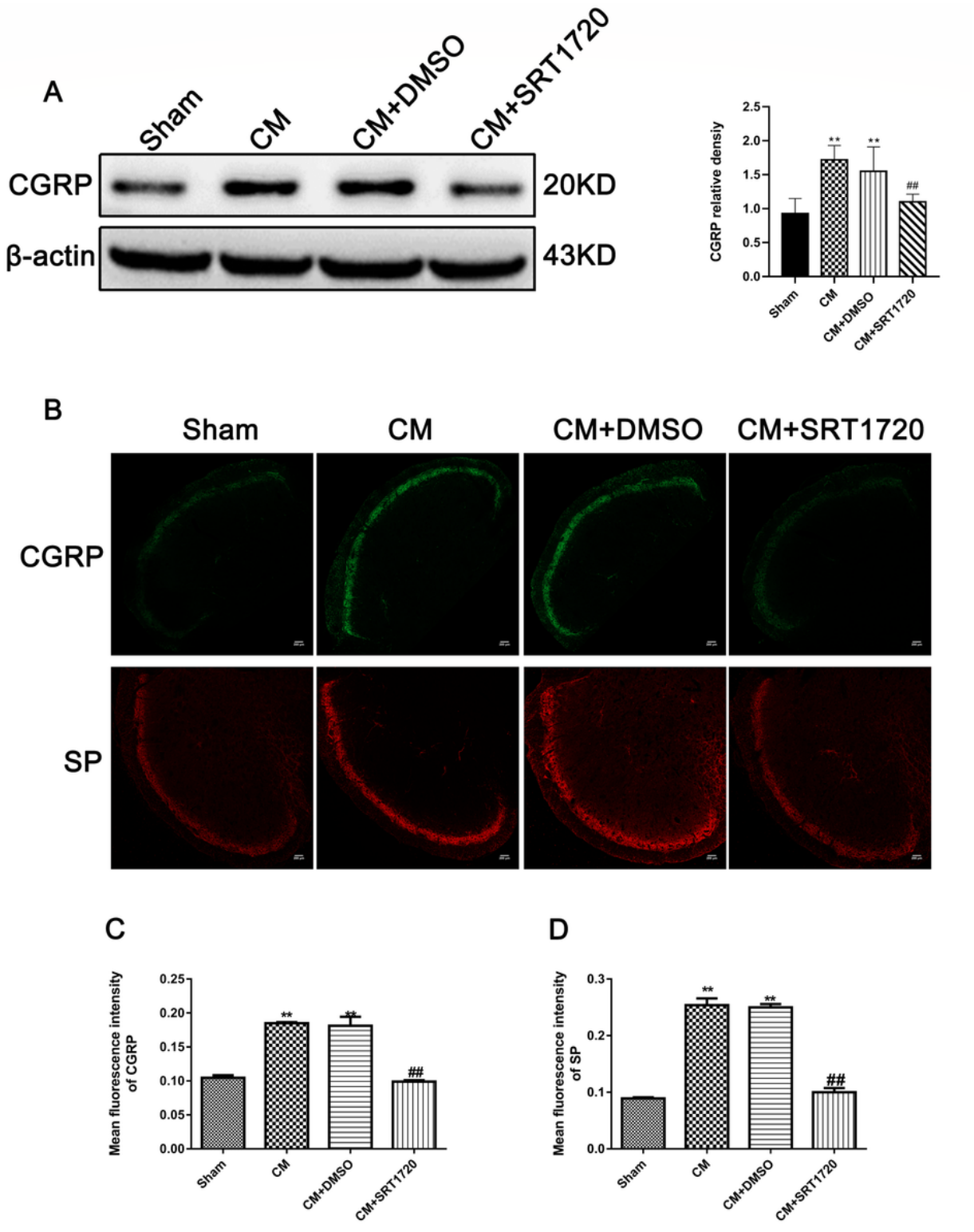

Figure 7

Expression of CGRP in the TNC and immunofluorescence staining for CGRP and SP in the TNC. A) The expression of CGRP was significantly increased in the CM group compared with the sham group, and no significant difference in the expression of CGRP was found between the CM and CM + DMSO groups. The expression of CGRP in CM rats was significantly decreased after administration of SRT1720 (n = 6 in each group, five images per animal, **P< 0.01 compared with the sham group, ##P< 0.01 compared with

the CM + DMSO group). B)The bilateral immunoreactivity of CGRP and SP in the TNC was investigated by immunofluorescence staining (the image shows only one side because no difference was found between the two sides).C) The fluorescence intensity of CGRP in the TNC was elevated in the CM groups compared with the sham group, and no significant difference in the expression of CGRP was found between the CM and CM + DMSO groups. SRT1720 treatment decreased the CGRP fluorescence intensity. D) The fluorescence intensity of SP in the TNC was elevated in the CM group compared with the sham group, and there was no significant difference in the expression of SP between the CM and CM + DMSO groups. SRT1720 treatment decreased the SP fluorescence intensity (n = 6 in each group, **P< 0.01 compared with the sham group, ##P< 0.01 compared with the CM + DMSO group, scale bar = 200 μm).