



## SIRT2 overexpression decreases remifentanyl-stimulated post-surgical hyperalgesia via microglia

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### ABSTRACT

Remifentanyl (Remi)-induced hyperalgesia is a serious but common postoperative clinical problem. Sirtuin 2 (SIRT2) is essential in the pathogenetic mechanisms of several neurological disorders. However, whether SIRT2 contributes to the modulation of Remi-induced postsurgical hyperalgesia (POH) is unknown. Here, we investigated the regulatory potential of SIRT2 in Remi-stimulated POH. A rat Remi-stimulated POH model was built by infusing Remi in the surgical incision. Mechanical allodynia and thermal hyperalgesia were separately assessed by paw withdrawal mechanical threshold (PWMT) and paw withdrawal thermal latency (PWTl) measurements. SIRT2 and binding adaptor molecule 1 (Iba1) protein expressions and localization in spinal cord samples were detected by western blot and immunofluorescence. The results revealed SIRT2 downregulation in the spinal cord of rats with Remi-stimulated POH. Intrathecal administration of the overexpression plasmid harboring SIRT2 remarkably relieved mechanical allodynia, along with thermal hyperalgesia in the model animals. Iba1 amounts were increased upon intraoperative incision or Remi infusion, and this effect was more pronounced upon combining both treatments. Furthermore, SIRT2 overexpression suppressed microglia activation in the spinal cord of model animals, and starkly relieved incision- and/or Remi-associated pronociceptive processes as well as spinal microglia activation. SIRT2 elevation relieved Remi-associated POH, likely by suppressing spinal microglia activation. Thus, SIRT2 could be a potent target for treating neuropathic pain.

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### Introduction

Remifentanyl (Remi) represents a newly discovered ultra-short-acting  $\mu$ -opioid receptor. Compared to fentanyl, its analgesic effect is better. Nevertheless, upon Remi treatment discontinuation, nociceptive stimulation develops more quickly and is more prominent. Hyperalgesia triggered by Remi is overtly more pronounced compared with that triggered through long-acting opioids, which not only significantly improves the postoperative pain score and the need for analgesia (1, 2), but also affects recovery post-surgically and hinders its popularization.

To date, many key mechanisms of postoperative hyperalgesia associated with Remi administration have been proposed. For instance, neuroimmune mechanisms (microglia activation) contribute to postsurgical hyperalgesia (POH). Microglia represents brain immune cells. Activated microglia can release pro-inflammatory molecules, containing cytokines, chemokines, reactive oxygen species (ROS), as well as nitric oxide (NO), in response to pathology or tissue injury (3), which leads to a reduced threshold in reaction to pain signals and elevated neuron excitability. During pain, proinflammatory molecules released by microglia in the nervous system have critical functions in pain sensitization. Animal experiments assessing peripheral inflammation, spinal cord injury as well and nerve injury have indicated spinal cord microglia are critical for central sensitization (4) along with disease-as-

sociated pain. The essential roles of microglia induction and released modulatory molecules associated with the introduction of nociceptive events in animals with experimental chronic pain have been demonstrated (5, 6). Ionized  $Ca^{2+}$ -binding adapter protein 1 (Iba1) is encoded by the Allograft inflammatory factor 1 (Aif1) gene, which belongs to a conserved, intracellular, and proinflammatory  $Ca^{2+}$ -binding adapter protein that is selectively expressed by microglia and macrophages and has been used as a microglial marker (7). Iba1 possesses actin-binding activity and is involved in membrane ruffling as well as phagocytosis of activated microglia (8). Recently published reports have demonstrated chemokines, hydrogen peroxide, cytokines, ROS, along further inflammatory factors are essential in opioid-stimulated POH. We previously confirmed spinal microglia is highly induced in Remi-associated hyperalgesia, with significantly increased expression levels of pro-inflammatory molecules containing IL-6, TNF- $\alpha$ , as well as p-NR2B (9), indicating spinal microglia overtly contributes to Remi-associated hyperalgesia. However, the underlying mechanism remains unclear.

Sirtuin 2 (SIRT2) attracts increasing attention for its critical function in microglia activation. According to the literature, SIRT2 is produced by microglia, as well as inhibits their induction as well as the release of inflammatory cytokines. At the same time, it also mediates neuroimmune reactions in central microglia (10). Cell culture

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and animal studies have demonstrated that SIRT2 suppression or knockout markedly increases microglia responses to proinflammatory molecules, as well as enhances the conversion of ROS and reactive nitrogen radicals (10, 11). In addition, microglia induction associated with lipopolysaccharide (LPS) and TNF- $\alpha$  starkly decreases SIRT2 phosphorylation (11). In addition, cell culture experiments reveal the introduction of wild-type SIRT2 into N9 microglia after induction with LPS and TNF- $\alpha$  significantly reduced levels of NO, IL-6, and CD40 (an activation biomarker), and these effects were associated with phosphorylation at S331 (11). Both cell culture and animal studies have certified the SIRT2 suppressor AK-7 markedly increases microglia induction by traumatic brain injury and upregulates inflammatory molecules (10, 12, 13). Lys310 acetylation of p65 is enhanced upon SIRT2 suppression in microglia, inducing the nuclear translocation of NF- $\kappa$ B, as well as regulating the transcription of inflammatory factors (10, 11). SIRT2 can modulate oxidative stress through its forkhead box protein O3 (FoxO3a) deacetylation, thereby enhancing FoxO3A-dependent manganese superoxide dismutase (MnSOD) transcription (14), representing a type of the most critical endogenous antioxidant enzyme that catalyzes ROS degradation. However, whether SIRT2 contributes to the modulation of Remi-associated POH is unknown.

Herein, we examined SIRT2's contribution to Remi-associated POH. A rat Remi-associated POH was established, and the potential and mechanism of SIRT2 overexpression in Remi-associated POH were assessed.

## Materials and Methods

### Animals

The Experimental Animal Center of Nanjing Drum Tower Hospital (Nanjing, China) provided adult male Sprague-Dawley (SD) rats ( $n=64$ ; 200 to 250 g). The rats were maintained at  $22\pm 2^\circ\text{C}$  under a 12-h light/12-h dark cycle, with rodent chow and water at will. Relevant guidelines and regulations were followed in this study, which was carried out after approval from the Experimental Animals Welfare and Ethics Committee of Nanjing Drum Tower Hospital.

### Establishment of a rat model of Remi-associated POH

Upon anesthesia using sevoflurane (Jiangsu Hengrui Pharmaceutical, China) by inhalation (induction, 3.5%; maintenance, 3.0%), the animals ( $n=32$ ) underwent subcutaneous administration of Remi (Yichang Humanwell Pharmaceutical) at 0.04 mg/kg (in 0.4 ml normal saline (NS)) at 0.8 ml/h with an infusion pump. Such treatment induces pronociceptive effects (9). Control animals underwent subcutaneous treatment with the same NS volume and flow rate ( $n=32$ ).

The operative incisions for postsurgical pain induction were implemented as reported in a previous work (15). In brief, right hind paw sterilization was carried out with 10% povidoneiodine before incision of skin and fascia. After longitudinal incision and retraction of the flexor muscle, the lesion was sutured, and erythromycin ointment was applied. The incision was initiated about 5 min post-treatment with Remi or NS. Control animals were treated by the same process but underwent no plantar incision.

### Intrathecal catheter implantation

Intrathecal catheters were implanted based on a previous study (16). In brief, after completion of anesthesia, a catheter was placed in the cisterna magna by advancing a 7.0 cm incision posterior to the lumbar enlargement. Then, catheter fixation was performed before suturing.

### Administration of recombinant lentiviruses

Recombinant lentiviruses-SIRT2, provided by Applied Biological Materials (GENE, Shanghai, China) was amplified in 293T cells, followed by purification by the double CsCl purification technique. For transfection,  $10^8$  plaque-forming units (PFU) adenoviruses were administered by injection using a microinjection syringe attached to an intrathecal catheter.

### Sample collection

One month following treatment with recombinant lentiviruses, euthanasia was carried out. Then, L4-L5 lumbar spinal cord samples were extracted to obtain dorsal root ganglia (DRG) for analysis.

### Animal grouping

Based on treatment, the animals were divided into the Sham+NS (sham operation + NS infusion), Inci+NS (incision + NS infusion), Sham+Remi (sham operation + Remi infusion), Inci+Remi (incision and Remi infusion), Sham+LV-control (sham operation + LV-control), Sham+LV-SIRT2 (sham operation + LV-SIRT2), Inci+Remi+LV-control (incision + Remi infusion upon LV-control administration) and Inci+Remi+LV-SIRT2 (incision + Remi infusion upon LV-SIRT2) groups.

For behavioral assays, 32 animals were randomized into the Sham+NS, Inci+NS, Sham+Remi, and Inci+Remi groups ( $n=8$ ) and assessed the day preceding the surgery (baseline values) and at different times following surgery. For immunoblot, 32 animals were randomized into the same groups as behavioral assays ( $n=8$ ), and sample collection was performed 2 days following surgery. For immunofluorescence, 16 animals were randomized into the Sham+LV-control, Sham+LV-SIRT2, Inci+Remi+LV-control, and Inci+Remi+LV-SIRT2 groups ( $n=4$ /group), and sample collection was performed at 2 postoperative days. Observations and analyses were performed in a blinded fashion.

### Nociceptive behavior assays

Paw withdrawal mechanical threshold (PWMT) measurement was carried out with von Frey filaments to assess mechanical allodynia. Rats placed in  $20\times 20\times 15$ -cm plastic boxes with a  $1\times 1$ -cm wire mesh bottom were acclimatized for 30 min. Von Frey filaments were utilized in a vertical manner to the plantar surface close to the wound of right hind paw for 6-8 s with adequate force. A positive response was reflected by quick paw withdrawal. Withdrawal responses were assessed at 5-min intervals. PWMT measurement was performed with sequential increase and decrease of stimulus strength (17). Five tests were performed at each stimulus strength, and  $\geq 3$  positive responses of the weakest force constituted PWMT. PWMT was measured thrice at every time point.

For thermal hyperalgesia assessment, paw withdrawal thermal latency (PWTL) measurement was performed with a biological research apparatus (Ugo Basile 37370,

Comerio, Italy) (18). The animals in glass-floored test cages underwent a 15-min acclimatization. A mean PWTL approximating 10 s was set up prior to testing, with a cut-off latency of 15 s. The infrared source was placed directly under an area next to the created wound. PWTL was the time elapsed from the onset of the infrared heat stimulus to paw withdrawal. Measurements were performed thrice at 5-min intervals, and values were averaged.

### Immunoblot

Western blots were implemented as previously described (19). Spinal cord (right dorsal part of L4–L5) specimens underwent homogenization with lysis buffer. Protein quantitation in lysates was assessed with the bicinchoninic acid (BCA) Protein Assay Kit (Sigma, USA). Equal quantities (50 µg) of total protein underwent separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transfer onto a nitrocellulose membrane. Blocking was implemented with 5% fat-free milk at ambient, followed by overnight incubation with primary antibodies targeting Iba1 (1:600, ab15690, Abcam) and SIRT2 (1:2000, ab211033, Abcam), respectively. Following three washes, the membrane underwent incubation with horseradish peroxidase-linked goat anti-mouse (1:5000, ab97040, Abcam) or goat anti-rabbit (1:5000, ab7090, Abcam) secondary antibodies (1 h at ambient). The electrochemiluminescence system (Millipore Immobilon) was utilized for detection. Immunoreactive bands were quantitated with Image-Pro Plus 6.0 (Media Cybernetics, USA), and data were normalized to β-actin expression.

### Immunofluorescence

Immunofluorescence was implemented as described before (20). L4–L5 spinal cord segments underwent 4% paraformaldehyde fixation and incubation with 30% sucrose. Transverse spinal sections (25 µm) underwent blocking with 10% goat serum in 0.3% Triton for 1 h at ambient, prior to overnight incubation (4°C) with anti-Iba1 primary antibodies (1:200, Abcam). Next, a 1-h incubation was carried out with Alexa Fluor 488 secondary antibodies (1:1500, Thermo Fisher) at ambient. A Leica multiphoton confocal microscope (Leica Microsystems, Wetzlar, Germany) was utilized for analysis.

### Statistical analysis

Statistical analysis was carried out with SPSS 15.0. Data was as mean ± standard deviation (SD). Behavioral data were compared by repeated-measures two-way analysis of variance (ANOVA), with post hoc Bonferroni test. Immunoblot data were compared by one-way ANOVA with post hoc Bonferroni multiple comparisons.  $P < 0.05$  was statistically significant.

## Results

### Effects of Remi on mechanical allodynia together with thermal hyperalgesia

For assessing whether Remi induces hyperalgesia, pain behavior alterations in different groups were tested. Baseline PWMT and PWTL were similar in the 4 groups (Figure 1). The Sham+NS group showed no marked nociceptive threshold changes in comparison with baseline values. In the Inci+NS group, operative incision resulted

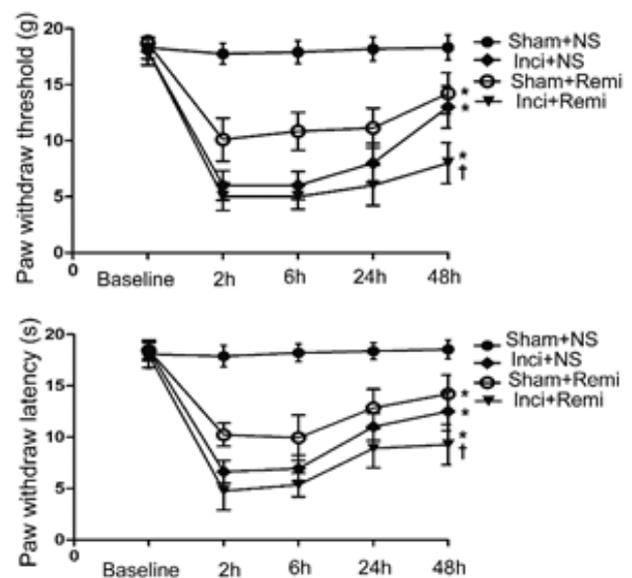
in enhanced mechanical allodynia along with thermal hyperalgesia 2 hours after operation, which were maintained for 2 days. In the Sham+Remi group, Remi infusion triggered mechanical allodynia, along with thermal hyperalgesia, as early as 2 h post-operation and lasted for 2 days. In the Inci+Remi group, Remi exacerbated incision-triggered mechanical allodynia together with thermal hyperalgesia relative to the Inci+NS group. These findings demonstrated Remi-induced hyperalgesia.

### Incision- and/or Remi-associated elevation of microglia biomarkers in the spinal cord

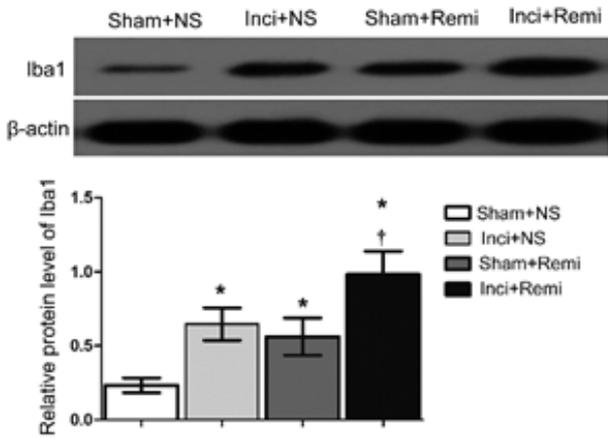
Iba1 is a sensitive marker associated with activated microglia (21). For the sake of assessing whether Remi-associated hyperalgesia is associated with microglia induction, immunoblot and immunofluorescence were carried out to detect Iba1 expression changes by microglia in spinal cord samples. Immunoblot revealed both incision and Remi administration as a single treatment resulted in elevated Iba1 amounts in comparison with the Sham+NS group (Figure 2). Next, Remi infusion exacerbated Iba1 elevation triggered by the incision in the Inci+Remi group. In agreement, immunofluorescence demonstrated that compared to the Sham+NS group, Iba1 amounts were slightly elevated in the Inci+NS and Sham+Remi groups while starkly elevated in the Inci+Remi group (Figure 3). The above findings indicated Remi might result in excessive microglia induction at the spinal cord level of healthy rats as well as animals with the surgical incision.

### Incision- and/or Remi-associated downregulation of SIRT2 in spinal cord specimens

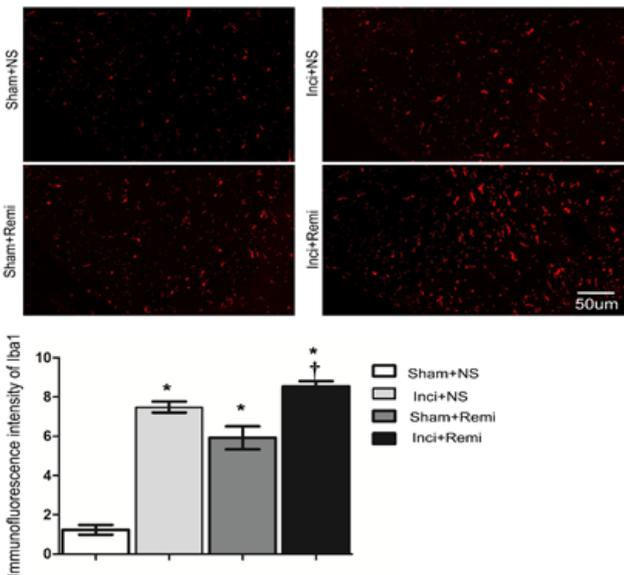
Immunoblot was performed for quantifying SIRT2 amounts in spinal cord specimens at 2 postsurgical days to further assess whether exacerbated microglia activation is associated with SIRT2. In contrast to the Sham+NS group, SIRT2 amounts presented slightly reduced in the Inci+NS and Sham+Remi groups, but starkly declined in the Inci+Remi group (Figure 4). The above findings sug-



**Figure 1.** Analysis of incision- and/or Remi-associated mechanical allodynia and thermal hyperalgesia (n=6). PWMT and PWTL were assessed at 1 day before (baseline) and at different times post-operation. \* $P < 0.05$ , compared with the Sham+NS group; † $P < 0.05$ , compared with the Inci+NS group.



**Figure 2. Analysis of Iba1 protein expression.** Iba1 protein level in the dorsal horn of L4–L5 spinal cord after induction by incision and/or Remi infusion 2 days post-surgery as assessed by immunoblot (n=4). \*P<0.05, compared with the Sham+NS group; †P<0.01, compared with the Inci+Remi group.



**Figure 3. Analysis of Iba1 protein expression and localization.** Immunofluorescence was carried out to assess Iba1 expression and localization in the dorsal horn of the L4–L5 spinal cord after operative incision and/or Remi infusion at 2 postsurgical days (n=4). Scale bar, 50 μm. \*P<0.05, compared with Sham+NS; †P<0.05, compared with the Inci+NS group.

gested Remi decreased SIRT2 protein amounts in spinal cord specimens from normal and surgically incised rats, revealing that Remi-induced hyperalgesia might be associated with SIRT2 downregulation.

### Co-expression of SIRT2 and Iba1 in the spinal cord

As shown in Figure 5, through immunofluorescence staining, SIRT2 was co-localized with Iba1 in the spinal cord 2 days after surgery. This finding suggested that SIRT2 might regulate microglia activation.

### SIRT2 overexpression relieves mechanical allodynia together with thermal hyperalgesia in model rats

For assessing the regulatory role of SIRT2 in Remi-associated postsurgical hyperalgesia in rats, SIRT2 was overexpressed in the animals by intrathecal injection of recombinant lentivirus expressing SIRT2 (LV-SIRT2). Then,

the impacts of SIRT2 elevation on thermal hyperalgesia together with mechanical allodynia were examined. We found SIRT2 overexpression markedly suppressed thermal hyperalgesia along with mechanical allodynia in rats (Figure 6), suggesting SIRT2 elevation inhibited Remi-associated postsurgical hyperalgesia.

### Overexpression of SIRT2 downregulates Iba1

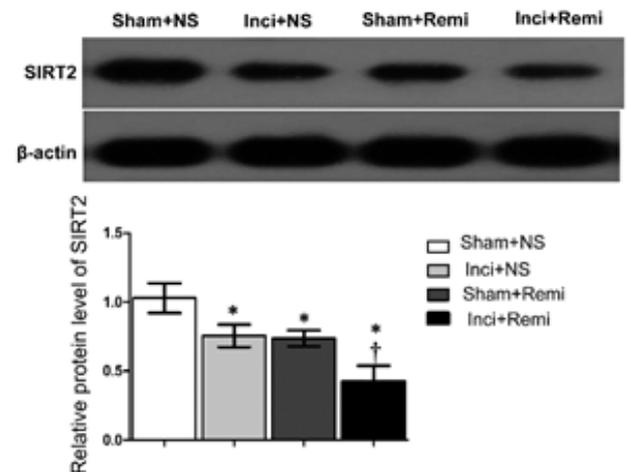
To assess the regulatory mechanism by which SIRT2 affects Remi-associated postsurgical hyperalgesia, the impact of SIRT2 elevation on Iba1 was examined. The results demonstrated that SIRT2 overexpression markedly reduced Iba1 protein amounts in the spinal cord of Remi-associated postsurgical hyperalgesia rats (Figure 7). These findings suggested SIRT2 overexpression decreased Iba1 expression, indicating SIRT2 could suppress microglia induction in Remi-associated postsurgical hyperalgesia.

### SIRT2 overexpression suppresses microglia activation

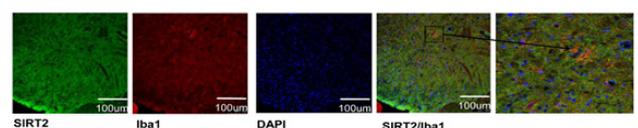
Immunofluorescence was next carried out to confirm SIRT2's function in microglia activation. Iba1 amounts were increased in the dorsal horn of the L4–L5 spinal cord in the Inci+Remi+LV-control group at the second postoperative day (Figure 8). In comparison with the Inci+Remi+LV-control group, Iba1 expression was slightly reduced in the Inci+Remi+Lv-SIRT2 group. The above finding proved SIRT2 suppressed microglia induction in Remi-associated postsurgical hyperalgesia.

### Discussion

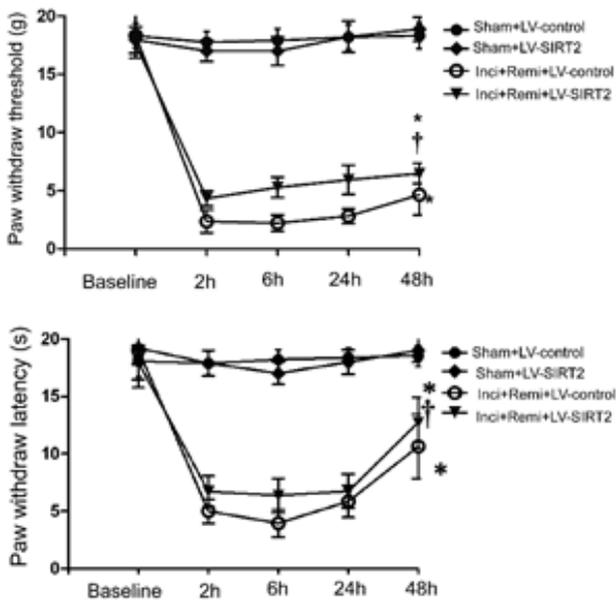
With the rising utilization of Remi in anesthesia, indi-



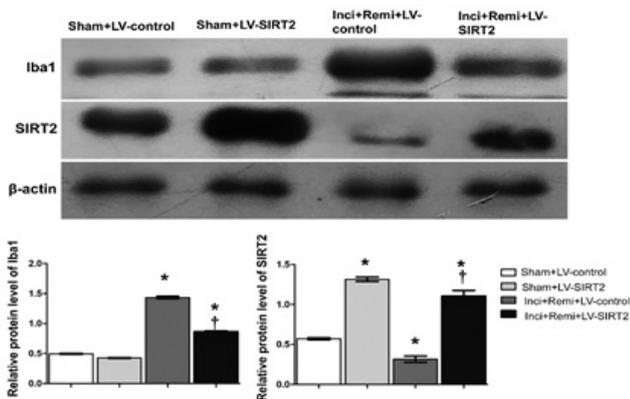
**Figure 4. Analysis of SIRT2 protein expression.** Immunoblot was conducted for measuring SIRT2 protein amounts in the dorsal horn of L4–L5 spinal cord post-treatment with incision and/or Remi infusion at 2 postoperative days (n=4). \*P<0.05, compared with the Sham+NS; †P<0.05, compared with the Inci+NS group.



**Figure 5. Analysis of SIRT2 and Iba1 co-localization.** SIRT2 and Iba1 expression in the dorsal horn of L4–L5 spinal cord upon surgical incision and Remi administration at 2 postoperative days as assessed by immunofluorescence staining (n=4).



**Figure 6. SIRT2 elevation relieves mechanical allodynia together with thermal hyperalgesia in model rats.** Mechanical allodynia was tested by detecting the paw withdrawal threshold after von Frey hair stimulation. Thermal hyperalgesia was tested by detecting paw withdrawal latency after radiant heat stimulation.  $N = 6$ . \* $P < 0.05$  vs Sham+LV-control group; † $P < 0.01$  vs Inci+Remi+LV-control group.



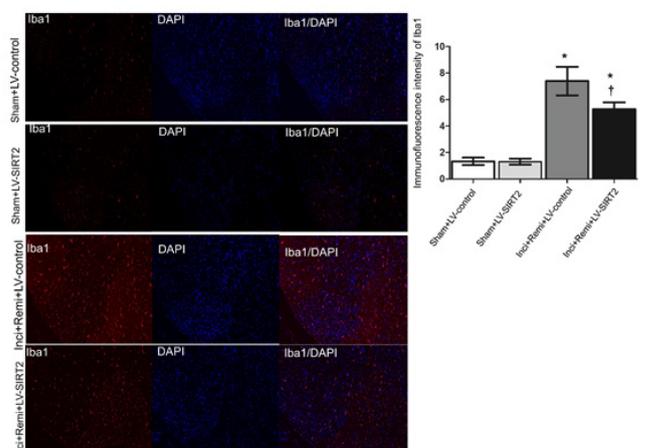
**Figure 7. SIRT2 overexpression decreases Iba1 expression.** DRG samples obtained from the L4–L5 lumbar spinal cord at 2 days postoperatively were examined by immunoblot for SIRT2 and Iba1 protein levels. \* $P < 0.05$  vs Sham+LV-control group; † $P < 0.01$  vs Inci+Remi+LV-control group.

viduals administered Remi for perioperative pain control may paradoxically promote the developed postsurgical pain. Remi (0.04 mg/kg) utilized in our study was in line with previously reported methods (9, 22). The above findings reveal that many treatment modalities, e.g., sevoflurane anesthesia, sham operation, as well as NS infusion, do not markedly affect pain behavior, corroborating our former reports (9, 22). Both incision and Remi administration significantly stimulated mechanical allodynia along with thermal hyperalgesia. Nevertheless, infusion of Remi perioperatively as a portion of general anesthesia markedly increased incisional-related pain as well as resulted in lengthened mechanical allodynia, along with thermal hyperalgesia. Former studies have investigated the mechanism of Remi-stimulated hyperalgesia. For instance, Yunan Peng et al have pointed out that COX-2 contributes to Remi-stimulated hyperalgesia related to ephrinB/EphB

signaling (23). Linlin Zhang et al have found that intrathecal injection of artesunate impairs Remi-stimulated hyperalgesia by down-regulating spinal mGluR5 expression in rats (24). However, the underlying mechanism of Remi-stimulated hyperalgesia is still required to be probed.

Mounting evidence suggests SIRT2 is important in many neurological diseases. For instance, Zhang and colleagues demonstrate that SIRT2 overexpression attenuates neuropathic pain as well as neuroinflammation by regulating the NF- $\kappa$ B pathway (25). Wang and collaborators show that, in the rat model of Parkinson's disease, inhibiting SIRT2 results in lessened striatal dopamine consumption, as well as improved antioxidant ability and behavioral abnormalities (26). In Alzheimer's disease, SIRT2 inhibition restores microtubule stability along with eliminating A $\beta$  oligomers. Additionally, SIRT2 suppression lowers steroid amounts, along with reducing the toxic potential of mutant Huntington protein in Huntington's disease (27). SIRT2 suppression induces antidepressant-like activities, and its increase alleviates depression (28, 29). Xie and co-workers show that SIRT2 downregulation protects against ischemic stroke in mice (30). Krey and collaborators demonstrate that SIRT2 knockout preserves neural function in a mouse model of stroke (31). However, Yuan and colleagues suggest that SIRT2 suppression results in anabolic blood-brain barrier damage together with traumatic brain damage in mice (10). Meanwhile, whether SIRT2 contributes to the modulation of opioid-associated post-surgical hyperalgesia, e.g., by Remi, remains unknown. As demonstrated above, the SIRT2 level was reduced in the DRG of Remi-treated POH rats. In addition, SIRT2 overexpression suppressed mechanical hypersensitivity and thermal hyperalgesia in the rat model.

It is currently admitted microglia, besides neurons, contribute to pain initiation and maintenance in several disorders (32). Evidence indicates microglia activation following pain and injury, which results in the release of multiple mediators, e.g., proinflammatory factors, to control neuronal activity as well as synaptic strength (33). Opioids also trigger microglia activation, which causes opioid-induced hyperalgesia. Although reports exploring the detailed mechanism of opioid-associated hyperalge-



**Figure 8. Analysis of Iba1 expression after SIRT2 overexpression.** Iba1 expression levels in the dorsal horn of L4–L5 spinal cord in the sham, Inci+Remi+LV-control and Inci+Remi+LV-SIRT2 groups at 2 postoperative days, as assessed by immunofluorescence ( $n = 4$ ). \* $P < 0.05$  vs Sham+LV-control group; † $P < 0.01$  vs Inci+Remi+LV-control group.

sia have mostly involved neuronal cells, growing evidence suggests microglia activation is tightly associated with the occurrence and maintenance of opioid-associated hyperalgesia (34). It has been documented that cathepsin S contributes to Remi-induced hyperalgesia in rats via microglia activation (35). Consistently, this work revealed that activated microglia in the spinal cord of rats were associated with Remi-associated hyperalgesia. Moreover, previous studies have indicated that SIRT2 is a suppressor of microglial activation and brain inflammation (11). Likewise, our study further confirmed that SIRT2 could suppress microglia activation to inhibit Remi-stimulated POH.

However, our study had some limitations. First, the animal samples in our study were small. Additionally, our study didn't explore whether SIRT2 regulated microglia activation via certain signaling pathways. Therefore, further studies should be carried out in the near future to perfect our study.

### Conclusion

Overall, SIRT2 was down-regulated in the spinal cord of rats with Remi-stimulated POH. SIRT2 overexpression alleviated Remi-associated hyperalgesia. Additionally, SIRT2 overexpressed inhibited Iba1 expression to suppress microglia activation. The current findings showed that SIRT2 overexpression inhibited Remi-stimulated POH via inhibiting microglia activation. Our study might provide a novel sight for Remi-stimulated POH treatment.

### Informed Consent

The authors report no conflict of interest.

### Availability of data and material

We declared that we embedded all data in the manuscript.

### Authors' contributions

ZW and BJ conducted the experiments and wrote the paper; JM and LY analyzed and organized the data; YL and MZ conceived, designed the study and revised the manuscript.

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