SIRT3 alleviates neuropathic pain by deacetylating FoxO3a in the spinal dorsal horn of diabetic model rats

Chenghua Zhou,[1] Yufeng Zhang,[1] Xiaowei Jiao,[1] Guizhi Wang,[1] Ruiyao Wang,[1] Yuqing Wu [2]

[1]Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, Xuzhou, China
[2]Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical University, Xuzhou, China

Correspondence to
Dr Yuqing Wu, Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical University, Xuzhou, China; xzmcyqwu@163.com

CZ and YZ contributed equally.

Received 29 July 2020
Revised 18 September 2020
Accepted 29 September 2020
Published Online First 30 October 2020

ABSTRACT

Background The underlying mechanisms of neuropathic pain remain unclear. This work aimed to investigate the role of Sirtuin3 (SIRT3), an nicotinamide adenosine dinucleotide+-dependent histone deacetylase, in the development of neuropathic pain induced by type 2 diabetes mellitus (T2DM) and to explore the associated mechanisms.

Methods Diabetic neuropathic pain (DNP) in rats was induced by high-fat diet/low-dose streptozotocin. The pain behaviors were examined using the von Frey and Hargreaves tests. The levels of SIRT3, manganese superoxide dismutase (MnSOD) and catalase (CAT) were determined using Western blot and RT-qPCR. The acetylation, phosphorylation and ubiquitination of forkhead box class O3a (FoxO3a) were analyzed by immunoprecipitation and Western blot.

Results SIRT3 expression and activity were significantly reduced in the spinal dorsal horn of DNP model rats. Overexpression of spinal SIRT3 reversed the pain hypersensitivity in the DNP model rats, but knockdown of spinal SIRT3 mimicked the pain effect, eliciting pain hypersensitivity in normal rats. Moreover, overexpression of spinal SIRT3 in DNP model rats increased the FoxO3a level and upregulated the antioxidant genes MnSOD and CAT by deacetylating FoxO3a and inhibiting FoxO3a phosphorylation and ubiquitination. Knockdown of spinal SIRT3 in normal rats decreased the FoxO3a level and downregulated MnSOD and CAT by inhibiting the deacetylation of FoxO3a and further increasing FoxO3a phosphorylation and ubiquitination.

Conclusions These results suggest that, by deacetylating FoxO3a and further reducing its phosphorylation, ubiquitination and degradation in the spinal dorsal horn, SIRT3 stabilizes FoxO3a protein and inhibits oxidative stress, resulting in pain alleviation in T2DM model rats.

INTRODUCTION

Neuropathic pain is a common clinical symptom characterized by spontaneous pain, hyperalgesia, and allodynia.[1] However, the underlying mechanisms of neuropathic pain remain unclear. Accumulating evidence has demonstrated that oxidative stress at the spinal level plays an important role in the development of neuropathic pain. In animal models of neuropathic pain, oxidative stress markers such as malondialdehyde are increased, while the activities of antioxidant enzymes such as superoxide dismutase and catalase (CAT) are reduced.[2 3] Moreover, free radical scavengers and antioxidants effectively alleviate pain behavior in neuropathic pain models.[3 4] Therefore, regulating the oxidative stress status may play an important role in finding new drug targets for the treatment of neuropathic pain.

Sirtuin3 (SIRT3), a kind of nicotinamide adenosine dinucleotide (NAD+)-dependent histone deacetylase (HDAC), is primarily localized in the mitochondria. Increasing amounts of evidence suggest that SIRT3 can control cell survival, metabolism and stress adaptive responses by inhibiting mitochondrial oxidative stress. For example, in hyperoxia-induced acute lung injury mice, overexpression of SIRT3 increases the protein levels and enzymatic activity of manganese superoxide dismutase (MnSOD) and inhibits oxidative stress.[5] In SIRT3 knockout mice, the production of reactive oxygen species (ROS) increases, and their oxidative stress status is enhanced.[6] In mouse hippocampal cells, SIRT3 knockdown exacerbates hydrogen peroxide (H_2O_2)-induced oxidative injury, while SIRT3 overexpression reduces the generation of ROS and lipid peroxidation following injury.[7] However, whether SIRT3 is involved in the development of neuropathic pain by regulating mitochondrial oxidative stress is unknown.

Transcription factors of the forkhead box class O (FoxO) family are important regulators of the cellular stress response. The mammalian FoxO family consists of FoxO 1, 3a, 4 and 6. Among these, FoxO3a is one of the most important members involved in cellular antioxidant defense.[8] The transcriptional activity of FoxO3a can be regulated by post-translational modifications such as phosphorylation, acetylation and ubiquitination. Moreover, different modifications may influence each other. It has been demonstrated that phosphorylation of FoxO3a increases its nuclear export and reduces its transcriptional activity, and meanwhile, phosphorylated FoxO3a facilities the ubiquitination and degradation of FoxO3a. In addition, acetylated FoxO3a is more sensitive to phosphorylation.[9 10] SIRT3 has been reported to regulate the transcriptional activity of FoxO3a by deacetylation. In endothelial cells exposed to hypoxia, increased SIRT3 mediates deacetylation of FoxO3a and further contributes to the reduction of FoxO3a phosphorylation, ubiquitination and degradation. Consequently, increased FoxO3a upregulates a

To cite: Zhou C, Zhang Y, Jiao X, *et al*. *Reg Anesth Pain Med* 2021;**46**:49–56.

set of FoxO3a-dependent mitochondrial antioxidant enzymes including MnSOD, peroxiredoxin (Prx) 3, Prx5 and thioredoxin 2 (Trx2).[11] In microglia, SIRT3 reduces cellular ROS levels by deacetylating FoxO3a, which transactivates antioxidant genes, CAT and MnSOD.[12] However, whether SIRT3 is involved in the development of neuropathic pain by deacetylating FoxO3a and upregulating antioxidant genes is unknown.

Therefore, in this study, we investigated the role of spinal SIRT3 in the development of neuropathic pain induced by type 2 diabetes mellitus (T2DM), and we further explored whether the role of SIRT3 in diabetic neuropathic pain (DNP) was mediated by deacetylating FoxO3a and upregulating antioxidant genes.

METHODS

Animals

Healthy male Sprague-Dawley rats (age 6–8 weeks) were maintained 4–5 per cage in specific-pathogen free conditions (23–25°C, 40%–50% relative humidity, 12/12 hours light/dark cycles), and provided with food and water ad libitum.

Diabetic model rats were fed with a high-fat diet (Slaccas Laboratory Animal Co., Shanghai, China) containing 7% (w/w) sugar, 10% (w/w) oil, 5% casein(w/w), 2% fishmeal (w/w), 2% maltodextrin (w/w), 0.1% methionine (w/w) and 74% common feed (w/w). After 8 weeks, the fasting triglyceride (TG) level and the fasting total cholesterol (TC) level were detected using a Triglyceride Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and a Total Cholesterol Assay Kit (Nanjing Jiancheng Bioengineering Institute). The fasting insulin level was detected using a Rat Insulin ELISA Kit (ExCell Biotech Co., Taicang, China). Insulin-resistant diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich Co., St Louis, Missouri, USA) at 30 mg/kg, after a 12 hours overnight fast. Five days after the injection of STZ, hyperglycemia was confirmed using a Blood Glucose Kit (Nanjing Jiancheng Bioengineering Institute) on fasting blood samples obtained from the inner canthus. Rats with fasting blood glucose values≥11.1 mmol/L were considered to be diabetic.

Behavioral test

The nociceptive threshold to mechanical stimulation and thermal stimulation were evaluated by the von Frey filaments and Hargreaves tests, respectively, as described in our previous report.[13] Briefly, for the von Frey filaments test, the rats were placed separately in transparent plastic cages on an elevated metal mesh floor. After a 30 min accommodation period, the plantar surface of the hind paws was perpendicularly stimulated with calibrated filaments (North Coast Medical, Morgan Hill, California, USA) ranging from 2.0 g to 15.0 g for 6–8 s. The 50% mechanical withdrawal threshold (MWT) was determined using the up–down method of Chaplan *et al*.[14] For the Hargreaves test, the rats were individually placed in chambers on an elevated glass platform (IITC Life Science, Woodland Hills, California). The high-intensity movable radiant heat placed underneath the glass was focused onto the plantar surface. The latency of paw withdrawal in response to the radiant heat source was recorded as the thermal withdrawal latency (TWL). An automatic cut-off of 25 s was employed to prevent tissue damage. All behavioral tests were performed by an experimenter blinded to the treatment groups.

Western blotting analysis

The frozen spinal cords were homogenized in a lysis buffer containing a cocktail of protease inhibitors and phenylmethylsulfonyl fluoride (Beyotime Biotech, Jiangsu, China). The supernatants were collected after centrifugation at 12 000g for 15 min at 4°C, and the protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Massachusetts, USA). Equal amounts of protein samples were separated by SDS-polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology, China) and transferred onto a nitrocellulose membrane. The membrane was subsequently incubated with the appropriate primary antibodies including anti-SIRT3 (Cell Signaling Technology, Beverly, Massachusetts, USA), anti-FoxO3a (Cell Signaling Technology), anti-p-FoxO3a (Cell Signaling Technology), anti-MnSOD (Abcam, Cambridge, UK), anti-CAT (Abcam) and anti-β-actin (Bioworld, Louis Park, USA), followed by incubation with the IRDye 800CW second antibody (Li-Cor, Lincoln, Nebraska, USA). An Infrared Imaging System (Gene Company Limited, Hong Kong, China) was applied to detect the immunoreactive bands.

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated using a TRIzol reagent kit (Invitrogen, Carlsbad, California, USA) and transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA). RT-qPCR analysis was performed using the Roche 480 LightCycler detection system with the GoTaq qPCR Master Mix Kit (Promega, Madison, Wisconsin, USA). Each sample was analyzed in triplicate. The PCR primers used were as follows: SIRT3 5'-GCG GCT CTA CAC ACA GAA CAT-3' (forward) and 5'-CAG GTT TCA CAA CGC CAG TA-3' (reverse); MnSOD 5'-AAG GAG AGT TGC TGG AGG CTA TC-3' (forward) and 5'-CTC CTT ATT GAA GCC AAG CCA G-3' (reverse); CAT 5'-GAG GAA ACG CCT GTG TGA GA-3' (forward) and 5'-TTG GCA GCT ATG TGA GAG CC-3' (reverse); β-actin 5'-CCC ATC TAT GAG GGT TAC GC-3' (forward) and 5'-TTT AAT GTC ACG CAC GAT TTC-3' (reverse). The relative mRNA expression levels were normalized to β-actin.

Assay of SIRT3 activity

SIRT3 activity was measured by a SIRT3 Activity Assay Kit (Abcam). Briefly, after isolation and extraction of protein from the spinal cords, the protein extract was purified by immunoprecipitation, in which rabbit anti-SIRT3 antibody (Cell Signaling Technology) and Protein A Agarose Beads (Cell Signaling Technology) were used. The reaction mixture contained Fluoro-Substrate peptide solution, SIRT3 assay buffer, NAD, Developer, ddH_2O and SIRT3-Protein A Agarose Beads, and NAD-dependent deacetylase activity was measured according to the manufacturer's instructions. Fluorescence intensity was read continuously for 60 min at 2 min intervals with excitation at 340 nm and emission at 460 nm using the Synergy 2 Microplate Reader (BioTek, Vermont, USA).

Double immunofluorescent staining

Animals were deeply anesthetized with chloral hydrate and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS). The lumbar spinal tissues were removed and postfixed with 4% paraformaldehyde overnight at 4°C, followed by dehydration with 30% sucrose (dissolved in PBS) for 5–7 days at 4°C. The spinal cords were sectioned transversely at 30 µm using a cryostat (Leica, Wetzler, Germany), and stored in frozen stock solution at −20°C. For double immunofluorescent staining, after being blocked in 5% normal donkey serum in PBS and 1% Triton-X 100, the sections were incubated

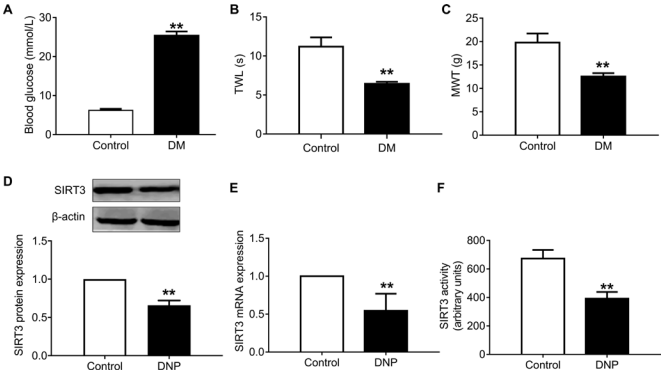

Figure 1 SIRT3 expression and activity are downregulated in the spinal dorsal horn of diabetic neuropathic pain (DNP) model rats. (A) The fasting blood glucose level in diabetic model rats and control rats 5 days after streptozotocin (STZ) injection. (B) Thermal withdrawal latency (TWL) and (C) mechanical withdrawal threshold (MWT) in diabetic rats and control rats 21 days after STZ injection. (D) Western blots for Sirtuin3 (SIRT3) protein, (E) real-time PCR for SIRT3 mRNA and (F) a SIRT3 fluorometric kit for SIRT3 activity in the spinal dorsal horn of rats were performed 21 days after STZ injection. All data are expressed as the mean±SD for each group, n=6. **P<0.01 versus control.

with the following primary antibodies: anti-GFAP (1:100, Millipore, Billerica, Massachusetts, USA), anti-Iba-1 (1:200, Abcam), and anti-NeuN (1:100, Abcam), respectively. After washing, the sections were then incubated with suitable secondary antibodies: Alexa Fluor 594 donkey antirabbit IgG (Life Technologies, Carlsbard, California, USA) or Alexa Fluor 594 donkey antigoat IgG (Thermo Fisher Scientific, Massachusetts, USA) in the dark for 2 hours, followed by incubation with fluorescein isothiocyanate (FITC) anti-SIRT3 (Biorbyt, Cambridge, UK) in the dark for 2 hours at 37°C. Cellular colocalization was examined with an LSM880 confocal laser-scanning microscope (Zeiss).

Intrathecal catheterization and viral injection

Intrathecal catheterization was performed as we described previously.[15] Briefly, the rats were anesthetized with 10% chloral hydrate and a PE-10 catheter was implanted between the L5 and L6 vertebrae. The catheter placement was verified by observing the transient hind paw paralysis induced by intrathecal injection of 2% lidocaine. The cannulated rats were allowed to recover for 3–4 days and were housed individually. Rat lentiviral vectors expressing SIRT3 (LV-SIRT3, 1×10^9 TU/mL) were obtained from GenePharma Co. (Suzhou, China) and were administered intrathecally for 5 consecutive days to the DNP model rats (daily from days 16–20 after STZ injection, 10 µL/d). Lentiviral vectors encoding SIRT3 siRNA (LV-SIRT3 siRNA, 1×10^9 TU/mL) with target sequence 5'-GGGCTGACGTGATGGCAGA-3' were obtained from GenePharma Co., and administered intrathecally for 5 consecutive days to the normal rats (10 µL/d).

Assays of FOXO3a acetylation and ubiquitination

FoxO3a lysine acetylation and ubiquitination were analyzed by immunoprecipitation (IP) of FoxO3a followed by Western blot using antiacetylated lysine (Ac-K) antibody and antiubiquitin (Ub) antibody. The frozen spinal cords were homogenized in a lysis buffer containing a cocktail of protease inhibitors and phenylmethylsulfonyl fluoride (Beyotime Biotech). The tissue extracts were centrifuged at 10 000 g at 4°C for 15 min, and the supernatant was used for IP. The protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific). A total of 0.5 mg protein extract was incubated with anti-FoxO3a antibody (Cell Signaling Technology) for 3 hours at 4°C. Then, 20 µL of precleared Protein A Agarose Beads (Cell Signaling Technology) were added and incubated at 4°C overnight for IP. The resulting immunoprecipitate was subjected to 8% SDS-polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology). The protein was transferred onto a nitrocellulose membrane and then blocked with 3% BSA. The membrane was incubated with the appropriate primary antibodies including anti-FoxO3a (Cell Signaling Technology), anti-Ac-K (Cell Signaling Technology), and anti-Ub (Abcam) at 4°C overnight. The membranes were washed with PBST, followed by incubation with the IRDye 800CW second antibody (Li-Cor). An Infrared Imaging System (Gene Company Limited) was applied to detect the immunoreactive bands.

Statistical analysis

Data are expressed as the mean±SD. Univariate comparisons were made against controls using Student's t-test. Multiple comparisons were analyzed using one-way analysis of variance followed by LSD post hoc test. P<0.05 was considered statistically significant.

RESULTS

T2DM model rats show significant pain hypersensitivity

In this study, we used high-fat diet/low-dose STZ to induce T2DM rat models. As shown in figure 1A, the fasting blood glucose level in the diabetic model rats was significantly elevated (P<0.01) after the injection of STZ compared with that in the control rats. Moreover, the diabetic model rats showed significantly decreased TWL (P<0.01) and MWT (P<0.01) on the 21st day after STZ injection, indicating that the diabetic model rats developed significant thermal hyperalgesia and mechanical allodynia (figure 1B and C).

Spinal SIRT3 expression and activity are downregulated in DNP rats

To investigate the underlying molecular mechanism of DNP, we determined the expression and activity of SIRT3 in the spinal dorsal horn of DNP model rats. We found that on the 21st day after STZ injection, both the SIRT3 protein (p<0.01) and mRNA levels (p<0.01) and SIRT3 activity (p<0.01) were decreased markedly in diabetic model rats compared with the control rats (figure 1D–F), suggesting that SIRT3 may be involved in the development of DNP.

SIRT3 is mainly localized in spinal dorsal horn neurons

To investigate the role of SIRT3 in the development of DNP, we first observed the cell types that express SIRT3 in the spinal dorsal horn using double immunofluorescent staining with specific markers of neurons, astrocytes and microglia. The results showed that SIRT3 mostly overlapped with NeuN, a neuronal marker, in the spinal dorsal horn. Nevertheless, SIRT3 hardly showed any overlap staining with either the astrocytic marker GFAP or the microglial marker Iba-1 (figure 2). These results suggest that SIRT3 localizes mainly in spinal dorsal horn neurons.

SIRT3 overexpression in the spinal dorsal horn alleviates pain hypersensitivity in DNP rats

To confirm the role of SIRT3 in the development of DNP, we observed the effect of SIRT3 overexpression in the spinal dorsal horn on pain behavior in DNP model rats. As shown

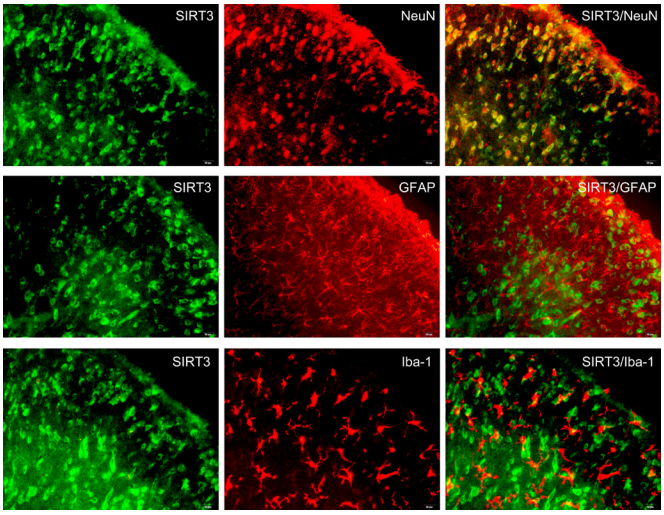

Figure 2 Cellular localization of Sirtuin3 (SIRT3) in the spinal cord of rats. Representative images with immunohistochemical staining for SIRT3 (green), neuronal nuclei (NeuN, red), glial fibrillary acidic protein (GFAP, red) and Iba-1 (red), and merged images for colocalization of SIRT3 and NeuN, SIRT3 and GFAP, SIRT3 and Iba-1. Scale bar: 20 µm.

in figure 3A–C, intrathecal injection of LV-SIRT3 induced a remarkable upregulation of SIRT3 protein, mRNA and activity in the spinal dorsal horn of DNP model rats, indicating a successful transfection of lentivirus into the spinal dorsal horn. Moreover, intrathecal injection of LV-SIRT3 significantly attenuated the thermal hyperalgesia and mechanical allodynia in DNP model rats (figure 3D and E). In addition, intrathecal injection of LV-SIRT3 caused a significant decrease in the level of blood glucose in DNP model rats (figure 3F). These results demonstrate that SIRT3 overexpression in the spinal dorsal horn by

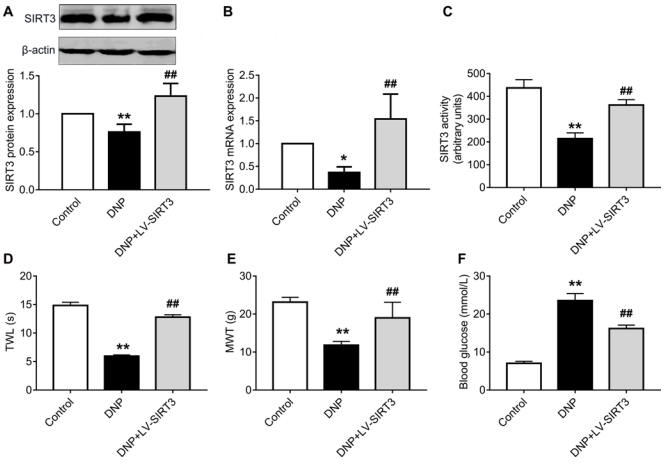

Figure 3 Sirtuin3 (SIRT3) overexpression in spinal dorsal horn alleviates pain hypersensitivity in diabetic neuropathic pain (DNP) model rats. LV-SIRT3 or control virus was injected intrathecally for 5 consecutive days from days 16 to 20 after streptozotocin (STZ) injection, and the pain behavior, blood glucose level and SIRT3 expression and activity were measured on day 21 after STZ injection. (A) Western blots for SIRT3 protein. (B) Real-time PCR for SIRT3 mRNA. (C) A SIRT3 fluorometric kit for SIRT3 activity. (D) Thermal withdrawal latency (TWL) for thermal hyperalgesia. (E) Mechanical withdrawal threshold (MWT) for mechanical allodynia. (F) Fasting blood glucose level. All data are expressed as the mean±SD for each group, n=6. *P<0.05, **P<0.01 versus control; ##P<0.01 versus DNP.

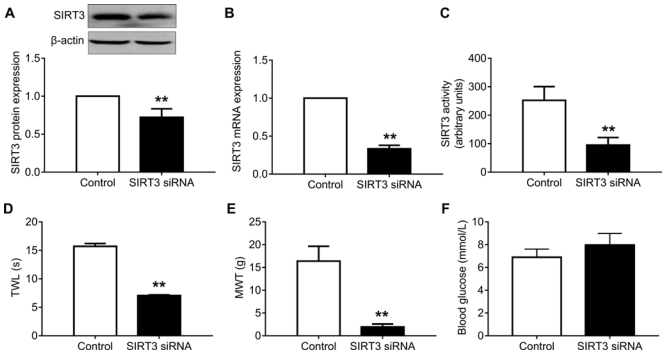

Figure 4 SIRT3 knockdown in the spinal dorsal horn induces pain hypersensitivity in normal rats. LV-SIRT3 siRNA or control virus was injected intrathecally for 5 consecutive days, and the pain behavior, blood glucose level, and SIRT3 expression and activity were measured on the sixth day. (A) Western blots for SIRT3 protein. (B) Real-time PCR for SIRT3 mRNA. (C) A SIRT3 fluorometric kit for SIRT3 activity. (D) Thermal withdrawal latency (TWL) for thermal hyperalgesia. (E) Mechanical withdrawal threshold (MWT) for mechanical allodynia. (F) Fasting blood glucose level. All data are expressed as the mean±SD for each group, n=6. **P<0.01 versus control.

intrathecal injection of LV-SIRT3 significantly alleviates pain hypersensitivity in DNP model rats.

SIRT3 knockdown in the spinal dorsal horn induces pain hypersensitivity in normal rats

To further determine if the absence of SIRT3 would influence pain behavior, we downregulated spinal SIRT3 by intrathecal injection of LV-SIRT3 siRNA in normal rats. The successful knockdown of SIRT3 was confirmed by the significant decrease of SIRT3 protein, mRNA and activity in the spinal dorsal horn of rats induced by LV-SIRT3 siRNA (figure 4A–C). Moreover, intrathecal injection of LV-SIRT3 siRNA induced thermal hyperalgesia and mechanical allodynia in normal rats (figure 4D and E). However, intrathecal injection of LV-SIRT3 siRNA had no influence on the level of blood glucose in normal rats (figure 4F). These results demonstrate that SIRT3 knockdown in the spinal dorsal horn by intrathecal injection of LV-SIRT3 siRNA could induce pain hypersensitivity in normal rats.

SIRT3 overexpression increases FoxO3a level and upregulates antioxidant genes by deacetylating FoxO3a in the spinal dorsal horn of DNP model rats

SIRT3 has been reported to mediate the deacetylation of FoxO3a.[12 16] Deacetylated FoxO3a further reduces FoxO3a phosphorylation, ubiquitination and degradation, thereby stabilizing the FoxO3a protein.[9 11] Therefore, in order to explore the underlying mechanisms for SIRT3 to alleviate DNP, we observed the effect of SIRT3 overexpression on FoxO3a acetylation, phosphorylation, ubiquitination and the FoxO3a level. As shown in figure 5, in the spinal dorsal horn of DNP model rats, the levels of FoxO3a acetylation, phosphorylation and ubiquitination were increased significantly, while the protein expression of FoxO3a decreased obviously. After intrathecal injection of LV-SIRT3 in DNP model rats, the levels of FoxO3a acetylation, phosphorylation and ubiquitination were decreased significantly, while the protein expression of FoxO3a increased markedly. These results suggest that overexpression of SIRT3 in the spinal dorsal horn of DNP model rats increases the FoxO3a level by deacetylating FoxO3a and further inhibiting FoxO3a phosphorylation, ubiquitination and degradation.

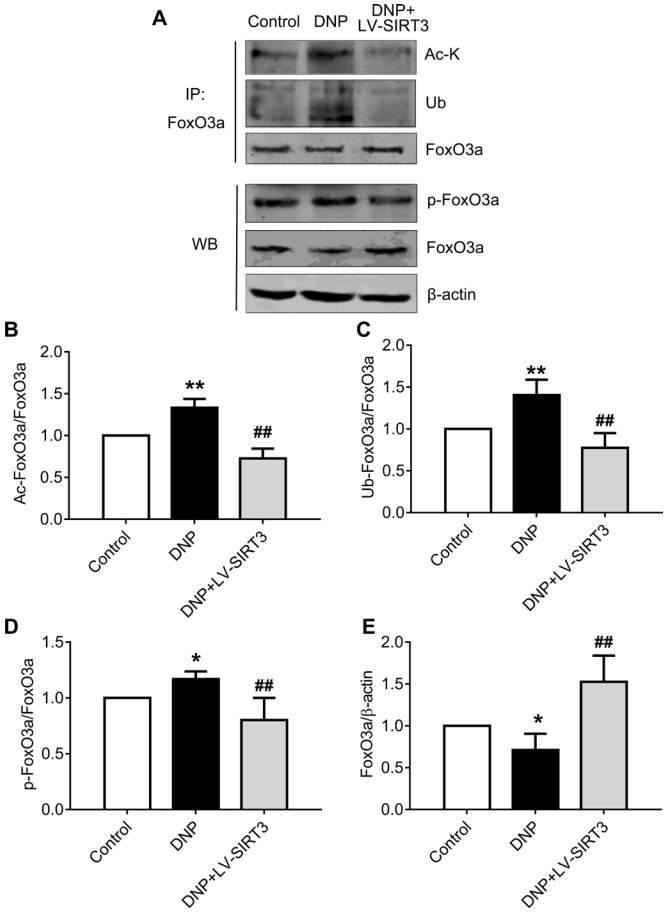

Figure 5 Sirtuin3 (SIRT3) overexpression increases the forkhead box class O3a (FoxO3a) level by deacetylating FoxO3a and further inhibiting FoxO3a phosphorylation and ubiquitination in the spinal dorsal horn of diabetic neuropathic pain (DNP) model rats. LV-SIRT3 or control virus was injected intrathecally for 5 consecutive days from days 16 to 20 after streptozotocin injection, and on day 21, the levels of FoxO3a acetylation (Ac-FoxO3a) and ubiquitination (Ub-FoxO3a) were measured by immunoprecipitation (IP) of FoxO3a followed by Western blot using antiacetylated lysine (Ac-K) antibody and antiubiquitin (Ub) antibodies, and the levels of total FoxO3a and FoxO3a phosphorylation (p-FoxO3a) were measured by Western blot. (A) Representative bands for Ac-FoxO3a, p-FoxO3a, Ub-FoxO3a and total FoxO3a. (B–E) Group data for Ac-FoxO3a, p-FoxO3a, Ub-FoxO3a and total FoxO3a. All data are expressed as the mean±SD for each group, n=6. *P<0.05, **P<0.01 versus control; ##P<0.01 versus DNP.

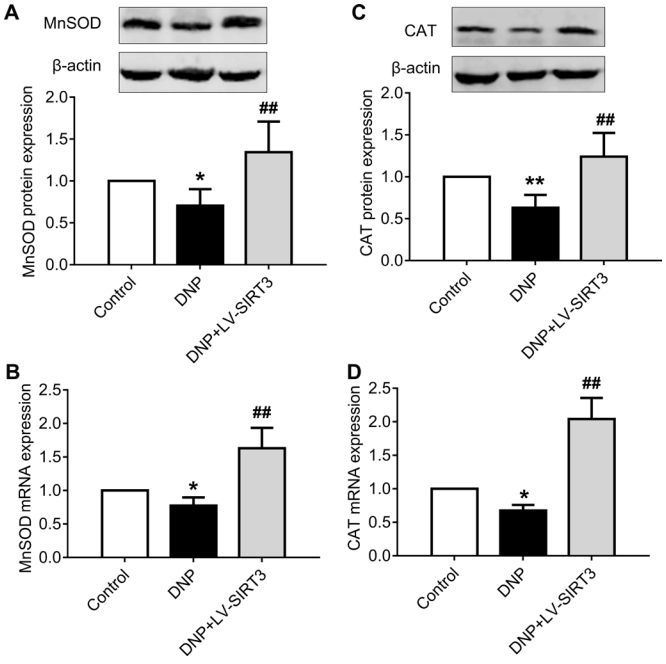

Figure 6 Sirtuin3 (SIRT3) overexpression upregulates antioxidant genes in the spinal dorsal horn of diabetic neuropathic pain (DNP) model rats. LV-SIRT3 or control virus was injected intrathecally for 5 consecutive days from days 16 to 20 after streptozotocin injection, and on day 21, the levels of manganese superoxide dismutase (MnSOD) and catalase (CAT) were measured by Western blot and real-time PCR. (A, C) Western blots for MnSOD and CAT proteins. (B, D) Real-time PCR for MnSOD and CAT mRNAs. All data are expressed as the mean±SD for each group, n=6. *P<0.05, **P<0.01 versus control; ##P<0.01 versus DNP.

It has been reported that an increased level of FoxO3a protein enhances antioxidant ability by activating the FoxO3a-dependent antioxidant genes, MnSOD and CAT.[16 17] Moreover, oxidative stress has been shown to be one of the important mechanisms in the pathogenesis of neuropathic pain.[18–21] Therefore, we observed the expression changes of MnSOD and CAT. As shown in figure 6, compared with the control rats, the decreased levels of MnSOD and CAT were found in the spinal dorsal horn of DNP model rats. However, intrathecal injection of LV-SIRT3 in the DNP model rats elevated the levels of MnSOD and CAT in the spinal dorsal horn. The above results suggest that overexpression of SIRT3 in the spinal dorsal horn of DNP model rats increases the FoxO3a level and upregulates antioxidant genes by deacetylating FoxO3a and inhibiting FoxO3a phosphorylation, ubiquitination and degradation.

SIRT3 knockdown decreases the FoxO3a level and downregulates antioxidant genes by inhibiting the deacetylation of FoxO3a in the spinal dorsal horn of normal rats

To confirm the effect of SIRT3 on the levels of FoxO3a and antioxidant enzymes, we downregulated SIRT3 in the spinal dorsal horn by intrathecal injection of LV-SIRT3 siRNA in normal rats. As shown in figure 7, after intrathecal injection of LV-SIRT3 siRNA, the levels of FoxO3a acetylation, phosphorylation and ubiquitination were increased significantly, while the protein expression of FoxO3a decreased obviously. In addition, the levels of the antioxidant enzymes MnSOD and CAT in the spinal dorsal horn decreased significantly in response to LV-SIRT3 siRNA (figure 8). These results suggest that knockdown of SIRT3 in the spinal dorsal horn of normal rats decreases the FoxO3a level and downregulates antioxidant genes by inhibiting deacetylation of FoxO3a and further increasing FoxO3a phosphorylation, ubiquitination and degradation.

DISCUSSION

Our present study demonstrates that SIRT3 function is significantly reduced in the spinal dorsal horn of DNP model rats. Viral overexpression of spinal SIRT3 reverses the pain hypersensitivity in DNP model rats, but viral knockdown of spinal SIRT3 mimics the pain effect, eliciting pain hypersensitivity in normal rats. Further study shows that SIRT3 stabilizes the FoxO3a protein and inhibits oxidative stress by deacetylating FoxO3a and further reducing its phosphorylation, ubiquitination and degradation in the spinal dorsal horn, resulting in the alleviation of pain in the T2DM model rats (figure 9). This study

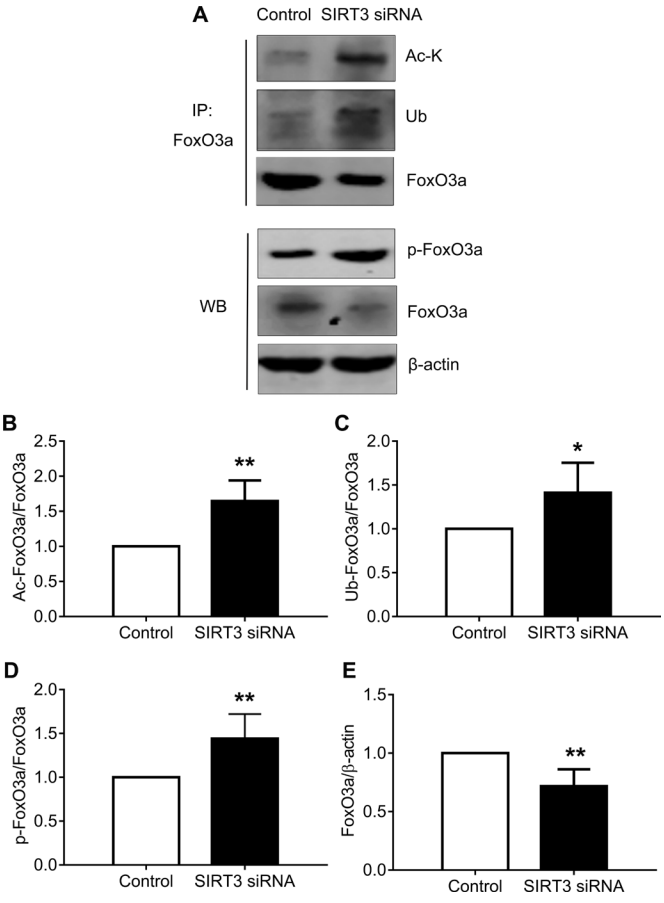

Figure 7 Sirtuin3 (SIRT3) knockdown decreases the forkhead box class O3a (FoxO3a) level by inhibiting the deacetylation of FoxO3a and further increasing FoxO3a phosphorylation and ubiquitination. LV-SIRT3 siRNA or control virus were injected intrathecally for 5 consecutive days, and on the sixth day, the levels of Ac-FoxO3a and Ub-FoxO3a were measured by immunoprecipitation (IP) and Western blot, while and the levels of total FoxO3a and p-FoxO3a were measured by Western blot. (A) Representative bands for Ac-FoxO3a, p-FoxO3a, Ub-FoxO3a and total FoxO3a. (B–E) Group data for Ac-FoxO3a, p-FoxO3a, Ub-FoxO3a and total FoxO3a. All data are expressed as the mean±SD for each group, n=6. *P<0.05, **P<0.01 versus control.

demonstrates that SIRT3 is a key epigenetic regulator in the development of DNP.

The underlying mechanisms of the development and maintenance of chronic pain remain unclear. There has been emerging evidence that epigenetic modifications, such as DNA methylation and histone modifications (eg, acetylation, ubiquitination and phosphorylation), can lead to long-term changes in gene expression of pronociceptive or antinociceptive genes, thereby contributing to the development and maintenance of chronic pain.[22 23] For example, it has been reported that HDAC-mediated histone deacetylation significantly represses the transcription of *Gad2*, which encodes glutamic acid decarboxylase 65, impairing γ-aminobutyric acid (GABA) synaptic inhibition in brainstem neurons that mediate descending pain modulation.[24] Our previous study demonstrated that SIRT1 attenuates neuropathic pain by epigenetic regulation of the metabotropic glutamate receptor 1 (mGluR1) and mGluR5 in type 2 diabetic rats.[13] These findings indicate that histone modification may be a key regulator in the development and maintenance of neuropathic pain.

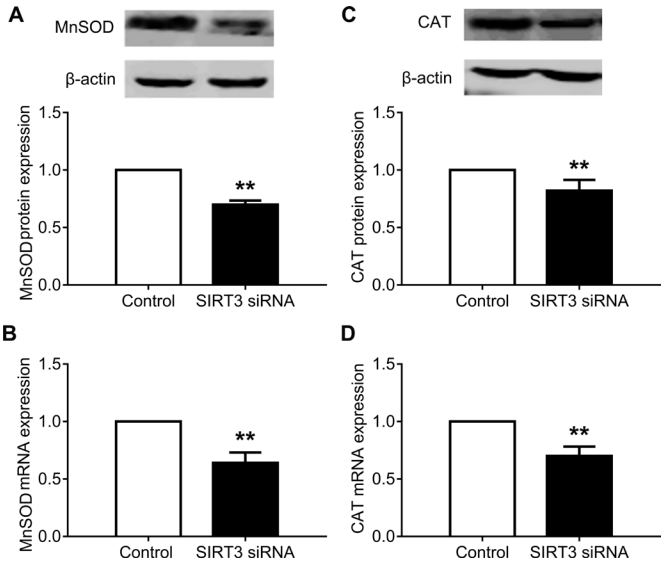

Figure 8 Sirtuin3 (SIRT3) knockdown downregulates antioxidant genes in the spinal dorsal horn of normal rats. LV-SIRT3 siRNA or control virus was injected intrathecally for 5 consecutive days, and on the sixth day, the levels of manganese superoxide dismutase (MnSOD) and catalase (CAT) were measured by Western blot and real-time PCR. (A, C) Western blots for MnSOD and CAT proteins. (B, D) Real-time PCR for MnSOD and CAT mRNAs. All data are expressed as the mean±SD for each group, n=6. **P<0.01 versus control.

SIRT3 is primarily localized in the mitochondria. Studies have shown that SIRT3 can activate some key enzymes through its deacetylating activity, thereby enhancing mitochondrial oxidative respiration, ensuring the stability of mitochondrial energy metabolism, and reducing the formation of ROS.[25 26] A growing body of evidence suggests that oxidative stress plays an important

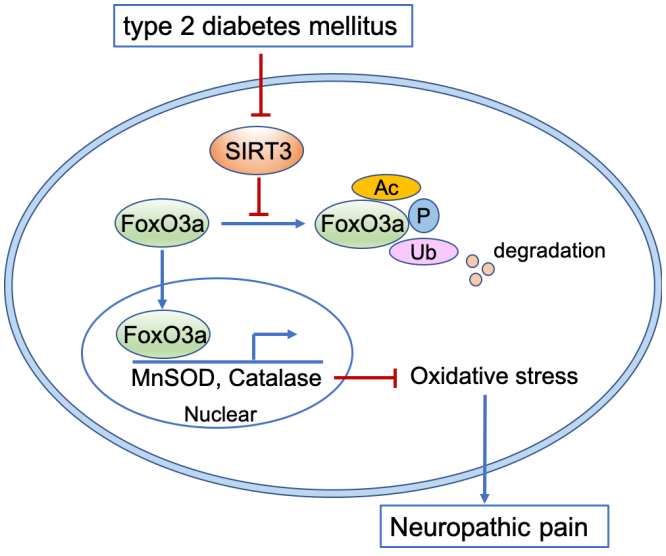

Figure 9 Schematic diagram demonstrating the role of sirtuin3 (SIRT3) in the development of diabetic neuropathic pain (DNP). During the development of DNP, the decreased function of SIRT3 induces an increase of FoxO3a acetylation, phosphorylation and ubiquitination. As a result, the protein expression of FoxO3a is decreased, and the transcription of antioxidant enzymes manganese superoxide dismutase (MnSOD) and catalase (CAT) is inhibited, leading to the enhancement of oxidative stress.

role in the development of neuropathic pain.[21 27] Therefore, we speculate that SIRT3 may be involved in the development of neuropathic pain by inhibiting oxidative stress.

To confirm this hypothesis, in this study, we used high-fat diet/low-dose STZ to induce T2DM rat models, and we found that the diabetic model rats developed significant mechanical allodynia and thermal hyperalgesia on day 21 post-STZ injection. This result is consistent with our previous report that on day 21 after STZ injection, rats have developed a significantly abnormal pain threshold.[13] In addition, the expression and activity of SIRT3 in the spinal dorsal horn of DNP model rats were both decreased. When we overexpressed spinal SIRT3 by LV-SIRT3, the thermal hyperalgesia and mechanical allodynia were improved in the DNP model rats. However, knockdown of spinal SIRT3 by LV-SIRT3-siRNA induced thermal hyperalgesia and mechanical allodynia in normal rats. These results suggest that SIRT3 is involved in the development of DNP.

It is known that FoxO3a plays a major role in limiting oxidative stress.[8] SIRT3 has been reported to regulate the transcriptional activity of FoxO3a by deacetylation.[12 16] Therefore, to explore the underlying mechanism of how SIRT3 attenuates DNP, we observed the role of SIRT3 in FoxO3a deacetylation and protein expression. Our results showed that overexpression of SIRT3 decreased the levels of FoxO3a acetylation and increased the protein expression of FoxO3a in DNP model rats, while knockdown of SIRT3 increased the level of FoxO3a acetylation, but decreased its protein expression in normal rats. As we know, histone deacetylation usually reduces the expression of substrate genes.[28] However, in this study, we found SIRT3 upregulated the level of FoxO3a, although it decreased FoxO3a acetylation. Therefore, we considered that SIRT3-mediated deacetylation of FoxO3a may regulate its protein expression by some indirect mechanisms. Indeed, it has been demonstrated that the transcriptional activity of FoxO3a can also be regulated by phosphorylation and ubiquitination. Moreover, acetylated FoxO3a is more sensitive to phosphorylation, and phosphorylated FoxO3a facilities its ubiquitination and degradation.[9 10] Therefore, we further observed the effect of SIRT3 on the levels of FoxO3a phosphorylation and ubiquitination. Our results showed that overexpression of SIRT3 reversed the enhanced phosphorylation and ubiquitination of FoxO3a in DNP model rats, while knockdown of SIRT3 induced an increase of phosphorylation and ubiquitination of FoxO3a in normal rats. The above results suggest that SIRT3 may stabilize the FoxO3a protein by deacetylating FoxO3a and further reducing its phosphorylation, ubiquitination and degradation.

It is well known that oxidative stress is a key player in neuropathic pain.[29] As an important nuclear transcriptional factor, FoxO3a has been reported to elevate the expression of the antioxidant genes MnSOD and CAT through directly binding to their promoter regions.[12 17] Therefore, we considered that the SIRT3-mediated FoxO3a deacetylation in DNP model rats might result in enhanced expression of MnSOD and CAT. In line with our prediction, the expression levels of MnSOD and CAT in the spinal dorsal horn of DNP model rats were downregulated significantly, and they were upregulated by SIRT3 overexpression. However, knockdown of SIRT3 by intrathecal injection of LV-SIRT3-siRNA induced the downregulation of MnSOD and CAT expression in the spinal dorsal horn of normal rats. These results suggest that SIRT3 may attenuate DNP in rats by inhibiting oxidative stress at the spinal level.

In this study, we also found that SIRT3 overexpression by intrathecal injection of LV-SIRT3 decreased the level of blood glucose in the DNP model rats, although their blood glucose was still higher than that of control rats. However, knockdown of spinal SIRT3 had no significant effect on blood glucose in normal rats. These results suggest that spinal SIRT3 may regulate blood glucose to some extent. Numerous studies have demonstrated that oxidative stress plays a pivotal role in the pathogenesis and development of diabetes mellitus,[30] and therefore, improvement of the oxidative stress status of the body by SIRT3 overexpression may be beneficial to decrease the level of blood glucose.

In conclusion, our present study demonstrates that the chronic neuropathic pain in diabetic model rats decreases the function of SIRT3 and results in an increase of FoxO3a acetylation, phosphorylation and ubiquitination, which causes decreased levels of FoxO3a protein and the antioxidant enzymes MnSOD and CAT. Overexpression of SIRT3 in the spinal dorsal horn of DNP model rats increases the level of FoxO3a and upregulates the antioxidant genes by deacetylating FoxO3a and further inhibiting FoxO3a phosphorylation, ubiquitination and degradation. These results suggest that SIRT3 activation may serve as a potential therapeutic strategy for chronic neuropathic pain.

Contributors CZ and YW designed the study and generated draft manuscript. YZ and CZ conducted the study and carried out the statistical analysis. XJ, GW and RW helped conduct the study and prepared experimental materials.

Funding This work is supported by the Natural Science Foundation of Jiangsu Province (BK20191464), and the Key Subject of Colleges and Universities Natural Science Foundation of Jiangsu Province (18KJA320008).

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Animal care and experimental procedures were approved by the Laboratory Animal Ethics Committee of Xuzhou Medical University (approval number: 201701w002).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

ORCID iD

Yuqing Wu http://orcid.org/0000-0001-8485-5951

REFERENCES

1 Holden JE, Pizzi JA. The challenge of chronic pain. *Adv Drug Deliv Rev* 2003;55:935–48.
2 Varija D, Kumar KP, Reddy KP, *et al*. Prolonged constriction of sciatic nerve affecting oxidative stressors & antioxidant enzymes in rat. *Indian J Med Res* 2009;129:587–92.
3 Naik AK, Tandan SK, Dudhgaonkar SP, *et al*. Role of oxidative stress in pathophysiology of peripheral neuropathy and modulation by N-acetyl-L-cysteine in rats. *Eur J Pain* 2006;10:573–9.
4 Mao Y-F, Yan N, Xu H, *et al*. Edaravone, a free radical scavenger, is effective on neuropathic pain in rats. *Brain Res* 2009;1248:68–75.
5 Tian YG, Zhang J. Protective effect of SIRT3 on acute lung injury by increasing manganese superoxide dismutase-mediated antioxidation. *Mol Med Rep* 2018;17:5557–65.
6 Jing E, Emanuelli B, Hirschey MD, *et al*. Sirtuin-3 (SIRT3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production. *Proc Natl Acad Sci U S A* 2011;108:14608–13.
7 Dai S-H, Chen T, Wang Y-H, *et al*. Sirt3 attenuates hydrogen peroxide-induced oxidative stress through the preservation of mitochondrial function in HT22 cells. *Int J Mol Med* 2014;34:1159–68.
8 Storz P. Forkhead homeobox type O transcription factors in the responses to oxidative stress. *Antioxid Redox Signal* 2011;14:593–605.
9 Boccitto M, Kalb RG. Regulation of Foxo-dependent transcription by post-translational modifications. *Curr Drug Targets* 2011;12:1303–10.
10 Wang Z, Yu T, Huang P. Post-Translational modifications of FOXO family proteins (review). *Mol Med Rep* 2016;14:4931–41.
11 Tseng AH-H, Wu L-H, Shieh S-S, *et al*. Sirt3 interactions with FOXO3 acetylation, phosphorylation and ubiquitinylation mediate endothelial cell responses to hypoxia. *Biochem J* 2014;464:157–68.
12 Rangarajan P, Karthikeyan A, Lu J, *et al*. Sirtuin 3 regulates FOXO3a-mediated antioxidant pathway in microglia. *Neuroscience* 2015;311:398–414.

13 Zhou C-H, Zhang M-X, Zhou S-S, *et al*. Sirt1 attenuates neuropathic pain by epigenetic regulation of mGluR1/5 expressions in type 2 diabetic rats. *Pain* 2017;158:130–9.
14 Chaplan SR, Bach FW, Pogrel JW, *et al*. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994;53:55–63.
15 Zhang Z, Ding X, Zhou Z, *et al*. Sirtuin 1 alleviates diabetic neuropathic pain by regulating synaptic plasticity of spinal dorsal horn neurons. *Pain* 2019;160:1082–92.
16 Sundaresan NR, Gupta M, Kim G, *et al*. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest* 2009;119:2758–71.
17 Kops GJPL, Dansen TB, Polderman PE, *et al*. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* 2002;419:316–21.
18 Yowtak J, Wang J, Kim HY, *et al*. Effect of antioxidant treatment on spinal GABA neurons in a neuropathic pain model in the mouse. *Pain* 2013;154:2469–76.
19 Ge Y, Jiao Y, Li P, *et al*. Coregulation of endoplasmic reticulum stress and oxidative stress in neuropathic pain and disinhibition of the spinal nociceptive circuitry. *Pain* 2018;159:894–906.
20 Gao X, Kim HK, Chung JM, *et al*. Reactive oxygen species (ROS) are involved in enhancement of NMDA-receptor phosphorylation in animal models of pain. *Pain* 2007;131:262–71.
21 Kim HK, Park SK, Zhou J-L, *et al*. Reactive oxygen species (ROS) play an important role in a rat model of neuropathic pain. *Pain* 2004;111:116–24.
22 Denk F, McMahon SB. Chronic pain: emerging evidence for the involvement of epigenetics. *Neuron* 2012;73:435–44.
23 Descalzi G, Ikegami D, Ushijima T, *et al*. Epigenetic mechanisms of chronic pain. *Trends Neurosci* 2015;38:237–46.
24 Zhang Z, Cai Y-Q, Zou F, *et al*. Epigenetic suppression of GAD65 expression mediates persistent pain. *Nat Med* 2011;17:1448–55.
25 Hirschey MD, Shimazu T, Goetzman E, *et al*. Sirt3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. *Nature* 2010;464:121–5.
26 Bause AS, Haigis MC. Sirt3 regulation of mitochondrial oxidative stress. *Exp Gerontol* 2013;48:634–9.
27 Gwak YS, Hassler SE, Hulsebosch CE. Reactive oxygen species contribute to neuropathic pain and locomotor dysfunction via activation of CaMKII in remote segments following spinal cord contusion injury in rats. *Pain* 2013;154:1699–708.
28 Seto E, Yoshida M. Erasers of histone acetylation: the histone deacetylase enzymes. *Cold Spring Harb Perspect Biol* 2014;6:a018713.
29 Sagalajev B, Wei H, Chen Z, *et al*. Oxidative stress in the amygdala contributes to neuropathic pain. *Neuroscience* 2018;387:92–103.
30 Ighodaro OM. Molecular pathways associated with oxidative stress in diabetes mellitus. *Biomed Pharmacother* 2018;108:656–62.