Neurochemical Research (2019) 44:450–464
https://doi.org/10.1007/s11064-018-2698-1

ORIGINAL PAPER

Suberoylanilide Hydroxamic Acid Triggers Autophagy by Influencing the mTOR Pathway in the Spinal Dorsal Horn in a Rat Neuropathic Pain Model

Xiang-Lan Feng[1] · Hong-Bo Deng[1] · Zheng-Gang Wang[1] · Yun Wu[1] · Jian-Juan Ke[1] · Xiao-Bo Feng[1]

Received: 28 July 2018 / Revised: 5 November 2018 / Accepted: 10 December 2018 / Published online: 17 December 2018

Abstract

Histone acetylation levels can be upregulated by treating cells with histone deacetylase inhibitors (HDACIs), which can induce autophagy. Autophagy flux in the spinal cord of rats following the left fifth lumber spinal nerve ligation (SNL) is involved in the progression of neuropathic pain. Suberoylanilide hydroxamic acid (SAHA), one of the HDACIs can interfere with the epigenetic process of histone acetylation, which has been shown to ease neuropathic pain. Recent research suggest that SAHA can stimulate autophagy via the mammalian target of rapamycin (mTOR) pathway in some types of cancer cells. However, little is known about the role of SAHA and autophagy in neuropathic pain after nerve injury. In the present study, we aim to investigate autophagy flux and the role of the mTOR pathway on spinal cells autophagy activation in neuropathic pain induced by SNL in rats that received SAHA treatment. Autophagy-related proteins and mTOR or its active form were assessed by using western blot, immunohistochemistry, double immunofluorescence staining and transmission electron microscopy (TEM). We found that SAHA decreased the paw mechanical withdrawal threshold (PMWT) of the lower compared with SNL. Autophagy flux was mainly disrupted in the astrocytes and neuronal cells of the spinal cord dorsal horn on postsurgical day 28 and was reversed by daily intrathecal injection of SAHA (n = 100 nmol/day or n = 200 nmol/day). SAHA also decreased mTOR and phosphorylated mTOR (p-mTOR) expression, especially p-mTOR expression in astrocytes and neuronal cells of the spinal dorsal horn. These results suggest that SAHA attenuates neuropathic pain and contributes to autophagy flux in astrocytes and neuronal cells of the spinal dorsal horn via the mTOR signaling pathway.

Keywords Histone deacetylase inhibitor · Autophagy flux · Mammalian target of rapamycin · Spinal dorsal horn · Neuropathic pain

Abbreviations

SAHA	Suberoylanilide hydroxamic acid
mTOR	Mammalian target of rapamycin
p-mTOR	Phosphorylated mTOR
PMWT	Paw mechanical withdrawal threshold
SNL	Spinal nerve ligation
HDACI	Histone deacetylation inhibitor
HDAC	Histone deacetylase
LC3	Microtubule associated protein light chain 3
CNS	Central nervous system

Introduction

Neuropathic pain is a common and refractory disease. International Associations for the Study of Pain (IASP) have defined it as a lesion or disease of the somatosensory nervous system that impairs the central nervous system and/or peripheral nervous system [1–4]. Trauma or disease of the somatosensory nervous system can lead to altered or disordered transmission of sensory signals to the spinal cord and brain [5], often resulting in chronic pain. Chronic pain greatly reduces the quality of life and increases the risk of developing comorbidities, such as depression and other mental health disorders [6]. In addition, neuropathic pain also significantly increases the economic burden on patients' families [6, 7]. However, more than two-thirds of neuropathic pain patients do not achieve sufficient pain relief [4]. The current clinical management of neuropathic pain is not optimal, and conventional analgesics, such as nonsteroidal

✉ Xiao-Bo Feng
lananesthesia@sina.com

[1] Department of Anesthesiology, Zhongnan Hospital of Wuhan University, No. 169 Donghu Rd, Wuhan 430071, Hubei, China

anti-inflammatory drugs and opioids, have moderate effects on neuropathic pain [8]. Therefore, finding new therapeutic targets is imperative. Histone deacetylase inhibitors (HDACIs) have recently been shown to ameliorate pain in different models of neuropathic pain [9, 10]. Mechanical and thermal hypersensitivity was attenuated only when pre-treatment with HDAC inhibitors [10].

Suberoylanilide hydroxamic acid (SAHA), one of the most interesting HDACIs, has been approved for the treatment of T cell lymphoma by the Food and Drug Administration (FDA) and can attenuate various pain-related behaviors in several pain models, including those of inflammatory pain and neuropathic pain [10–14]. Recent findings regarding epigenetic changes in the spinal cord during chronic pain have provided new guidelines for advanced treatments. The term epigenetics can lead to heritable changes in gene function without any changes in DNA sequence. Epigenetic mechanisms include DNA methylation, histone modifications, and microRNAs. HDACIs interfere with the epigenetic process of histone acetylation and have been shown to ease neuropathic pain [10, 13, 15]. Intrathecal pre-treatment with HDAC inhibitors have been already shown to alleviate to mechanical and thermal hypersensitivity in models of neuropathic pain [10].

Studies have shown that histone acetylation plays an important role in regulating autophagy; the cellular acetylation level can be upregulated by treating cells with HDACIs such as trichostatin A (TSA), and SAHA can induce autophagy [16]. Macroautophagy (hereafter called autophagy) degrades malfunctioning or damaged proteins, organelles, macromolecular complexes, and foreign bodies within lysosomes [17]. Recently, autophagy was also shown to participate in the progression of the pathology of neuropathic pain and is being exploited to be a possible therapeutic target [18, 19]. Autophagy is a highly dynamically regulated process that includes (i) the initiation of autophagy; (ii) the formation and expansion of an isolation membrane, which is also called a phagophore; (iii) the formation of the autophagosomes, which is a double-membrane structure containing phagophores; (iv) the fusion of autophagosomes with lysosomes; and (v) the degradation of autolysosomes [20], and this process is called autophagy flux. Impaired autophagy flux in the spinal cord of mice following the left fifth spinal nerve ligation (SNL) has been reported to be involved in the progression of neuropathic pain [21, 22]. A disruption of autophagy in Schwann cells has also been investigated [23]. In addition, autophagy activity was impaired in spinal GABAergic interneurons and glial cells after peripheral nerve trauma [21, 23, 24], suggesting that the disruption of autophagy might contribute to neuropathic pain. Based on the previous studies described above, how HDACIs are implicated in neuropathic pain remains poorly understood. The role of HDACIs and autophagy in neuropathic pain remains to be further explored.

The mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, has already been confirmed to be a key negative regulator of autophagy [25, 26]. Several lines of evidence have recently proven that SAHA can induce autophagy via the mTOR pathway in some types of cancer cells, including breast cancer cells, glioblastoma stem cells, and endometrial stromal sarcoma cells [25, 27, 28]. mTOR can be activated under many circumstances, such as in the presence of neurotransmitters, trophic factors, mitogens, and hormones [29]. Moreover, mTOR is activated in the spinal cord or spinal dorsal horn in models of neuropathic pain [30, 31] and in many cancer models [25, 27]. In turn, activated mTOR has effects on many physiologic and pathological processes in the nervous system [29], and the activated mTOR complex leads to impairment of autophagy activities. Recently, mTOR pathways have been demonstrated to be involved in the progression of neuropathic pain [32–34]; however, the cell type specificity involved in this process remains controversial.

There are three main types of glial cells in the central nerve system (CNS): microglia, astrocytes, and oligodendrocytes [35]. Glial cell proliferation and activation are involved in the development of neuropathic pain [36]. It has been extensively researched and accepted that astrocytes result in the generation and maintenance of neuropathic pain [35, 37]. Here, we examined the levels of autophagy and autophagic flux by western blot and immunohistochemistry following intrathecal continuous fusion of SAHA in a rat model of neuropathic pain. We further detected the levels of mTOR and its active (phosphorylated) forms (p-mTOR) in the ipsilateral spinal dorsal horn. Then, we used double immunofluorescent histochemistry staining to detect the cellular localization of autophagy markers. Transmission electron microscopy (TEM) is the only tool that can reveal the morphology of autophagic structures at a nm-scale resolution TEM can show these structures in their natural environment and position among other cellular components, allow their exact identification, and support quantitative studies if the rules of proper sampling are followed [38]. We also analyzed the cell autophagy phase by TEM. Finally, we sought to elucidate the signaling pathway of SAHA induction autophagy in SNL. In this study, SNL was conducted to establish a model of neuropathic pain. The aim of our study was to address (1) whether spinal astrocyte autophagy impairment leads to the development and maintenance of neuropathic pain; (2) whether SAHA treatment can contribute to autophagy flux, which mainly occurs in spinal astrocytes to attenuate SNL-induced neuropathic pain; and (3) whether the mTOR signaling pathway is responsible for SAHA treatment following SNL.

Materials and Methods

Animals and Surgery

One hundred and ten adult male Sprague–Dawley rats (200–220 g) used in the experiments were purchased from the Center for Animal Experimental of ZhongNan Hospital of WuHan University (WuHan, China). The rats were housed under a 12-h day-night cycle at a room temperature of 21 ± 2 °C, with free access to a standard laboratory chow diet and water. All rats were acclimated for 5 days before experimentation. All procedures were approved by the Animal Experimental Committee and of WuHan University, and all research adhered to the Guide for the Care and Use of Laboratory Animals (National Institutes Health).

Intrathecal Catheter Implantation

Rats were anesthetized with 2–2.5% isoflurane. A premeasured length of sterile polyethylene tube (PE-10 catheter, 8 cm) was threaded into the lumbar subarachnoid space between L4 and L5 and advanced approximately 3–4 cm at the level of the spinal cord lumbar enlargement sections as previously described [39]. To confirm that the catheter was inserted successfully, a suitable volume of 2% lidocaine (20 μl) was administered intrathecally. The rats became immediately immobilized due to paralysis and recovered from this condition within 30 min [40].

Drugs and Administration

All rats were randomly and equally assigned to nine groups (n = 12 each): a naïve group (naïve); a sham group receiving vehicle intrathecal injection (v-sham); a sham group receiving a low dose of SAHA intrathecal injection (l-sham); a sham group receiving a high dose of SAHA intrathecal injection (h-sham); a sham group receiving rapmycin intrathecal injection (r-sham); an SNL group undergoing left fifth lumber spinal nerve ligation receiving vehicle intrathecal injection (v-SNL); a rapamycin group (r-SNL); an SNL group receiving a low dose of SAHA (n = 100 nmol) (l-SNL); and an SNL group receiving a high dose of SAHA (n = 200 nmol) (h-SNL). All sham group rats underwent a surgical process without any nerve ligation. SAHA (A4084, APExBIO, USA) and rapamycin (HY-10219, MedChemExpress, USA) for pretreatment were dissolved in 30% dimethyl sulfoxide (DMSO) in saline solution and intrathecally injected immediately after implantation. SAHA (100 nmol/day or 200 nmol/day, 10 μl) or rapamycin (0.1 μg or 10 μl) were intrathecally injected and the dose and timing of drug administration were according to previously described methods [10, 11, 41]. Thus, SAHA and rapamycin were intrathecally injected for 12 days starting 5 days before SNL and continuing until 7 days after surgery.

SNL-Induced Neuropathic Pain

Nerve injury surgery was anesthetized with 2–2.5% isoflurane after continuous intrathecal injection of SAHA for 5 days. All rats recovered well from implantation and had no signs of wound infection. Nerve ligation in rats was processed as previously described [42]. Briefly, a small incision was made at the L4–S1 level, and the left L6 transverse process was removed to expose the L4 and L5 spinal nerves. Then, the left L5 spinal nerve was carefully isolated, lifted slightly and ligated with a 4–0 suture. In the sham group, rats underwent the same surgical procedures with no nerve ligation. Finally, the wound was closed. Any rats with neurological behavior deficits or that died during the study were excluded.

Behavioral Test

All the following tests were carried out by the same researcher who was blinded to the animal group assignments. The paw withdrawal mechanical threshold (PMWT) was measured using a series of calibrated von Frey filaments (Noah Coast, USA), ranging from 0.4 to 26 g (0.4, 0.6, 1, 1.4, 2, 4, 6, 8, 10, 15 and 26 g). The 2-g stimulus was used first, and the force was slowly adjusted upward or downward according to Dixon's up and down method [43]. Each non-narcotic rat was habituated to an inverted individual plastic cage (11 cm × 13 cm × 24 cm) with a wire mesh platform. Briefly, von Frey filament needles were applied vertically to the lateral plantar surface of the left (ipsilateral to nerve injury) hindpaw to determine the stimulus intensity required to elicit a paw withdrawal response. Significant hindpaw withdrawal reflections were recorded as the PMWT values before catheter implantation (henceforth called the baseline value, BL), 1 day before SNL surgery (-1 day) and on days 1, 3, 5, 7, 14, 21 and 28 after SNL surgery. Prior to testing, animals were habituated to this test environment for approximately 20 min, and each test was repeated 10 times on the same paw in 5-min intervals to obtain 50% PMWT values.

Western Blot Analysis

At 28 days after surgery, rats were deeply anesthetized with 2–2.5% isoflurane and rapidly sacrificed. The ipsilateral spinal lumbar segments (L4–L5) in the different groups were rapidly removed and frozen at − 80 °C. Tissues were rinsed with phosphate-buffered saline (PBS, pH 7.4) 2–3 times to remove blood, and then the spinal cord tissue was homogenized in a homogenizer containing homogenization

buffer and centrifuged at 14,000×*g* for 15 min at 4 °C. Total protein content was determined in the supernatants using a bicinchoninic acid (BCA, AS1086, ASPEN) protein assay kit following the manufacturer's instructions. For western blot analysis, each sample (40 μg, tissue) was separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15%) and transferred onto PVDF membranes. After blocking for 1 h at room temperature in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% nonfat milk, the membranes were incubated overnight at 4 °C with the primary antibody directed against the protein of interest. After washing three times with TBST, an appropriate HRP-conjugated goat anti-rabbit IgG (H + L) (1:10,000, AS1107, ASPEN), was applied for 1 h at room temperature. Peroxidase activity was visualized using an electrochemiluminescence (ECL) western blotting detection kit (ECL, AS1059, ASPEN), and images were captured. Signal intensity was measured using Quantity One software (Bio-Rad, Hercules, CA). For quantitative analysis, the LC3, Beclin-1, P62, mTOR and p-mTOR signals of each sample were normalized to the corresponding GAPDH signal. The following primary antibodies and dilutions were used: anti-LC3 (1:1000, #4108, Cell Signaling Technology, USA), anti-Beclin-1 (1:2000, #3738, Cell Signaling Technology, USA), anti-P62 (1:2000, ab109012, Abcam, UK), anti-mTOR (1:500, #2983, Cell Signaling Technology, USA), anti-p-mTOR (1:500, #5536, Cell Signaling Technology, USA) and anti-GAPDH (1:10,000, ab37168, Abcam, UK).

Immunohistochemistry and Double Immunofluorescence Staining

Mice were anesthetized with 2–2.5% isoflurane at 28 days after surgery. Approximately 150 or 200 ml of 0.9% saline was perfused through the ascending aorta, followed by 500 ml of 0.01 M PBS (pH 7.3, AS1025, Aspen China), which contained 4% paraformaldehyde and 2% picric acid. The L4-L5 spinal segments were removed and fixed in the same fixative at 4 °C overnight and then embedded in paraffin and blocked sections were cut (10 μm) with a microtome and then mounted onto slides.

The paraffin-embedded tissues were deparaffinized and rehydrated using a series of alcohol concentrations. The antigen was retrieved with EDTA (AS1016, Aspen, WuHan, China) in a microwave vacuum histoprocessor (p70D20p-TF, Galanz company, GuangDong, China) at a controlled temperature of 121 °C for 15 min. Hydrogen peroxide (3%) was used to block endogenous peroxidase activity. Then, the sections were rinsed in 0.01 M PBS three times (5 min each) and blocked with 5% bovine serum albumin (BSA, 10735078001, Roche) for 20 min at room temperature. The tissue slices were incubated overnight at 4 °C with rabbit anti-LC3 (1:200, ab12805, Abcam, UK), Beclin-1 (1:100, ab207612, Abcam, UK), P62 (1:200, ab56416, Abcam, UK), mTOR (1:400, ab2732, Abcam, UK) and p-mTOR (1:400, ab84400, Abcam, UK). The slices were incubated for 50 min with corresponding secondary antibodies: an appropriate HRP-conjugated goat anti-rabbit IgG (H + L) (1:200, AS1107, ASPEN). Immunostaining was visualized with diaminobenzidine (DAB, PAB180021, Bioswamp).

To simultaneously describe the presence of a pair of antigens in the same location, rabbit LC3, Beclin-1 and P62 were used with antiglial fibrillary acidic protein (GFAP, 1:500, ab7260, Abcam, UK), antiIba-1(1:200, ab15690, Abcam) and antiNeuN (1:150, ab104224, Abcam, UK) monoclonal antibodies. Slices were immunoreacted for LC3, Beclin-1, P62, mTOR and p-mTOR as described above. Sections were then further processed for GFAP, Iba-1, NeuN immunocytochemistry and the corresponding secondary antibody, Cy3-conjugated goat antirabbit antibody (1:50, AS-1109, ASPEN), and FITC-conjugated goat antimouse antibody (1:50, AS-1112, ASPEN), and counterstained with 4, 6-diamidino-2-phenylindole (DAPI, AS1075, ASPEN).

The number of immunoreactive (IR) LC3, Beclin-1, P62 immune reactive (IR) cells were counted in six slices of the lumber spinal dorsal horn for each group. The integral optical density (IOD SUM) of the immune reactive (IR) cell was measured by Image-Pro Plus software within a defined area of interest on the spinal dorsal horn.

Transmission Electron Microscopy

The mice were anesthetized with 2–2.5% isoflurane at 28 days after surgery and perfused with 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4). L4–L5 segments of the spinal cord dorsal horn were removed and fixed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.2) overnight and then postfixed in 1% osmium tetroxide for 1 h. The tissues were dehydrated with acetone and embedded in an epoxy resin. Ultrathin sections were cut, stained with 3% lead citrate, and examined via TEM.

Statistical Analysis

Unless otherwise stated, all values are presented as the mean ± standard deviation (SD). Data were collected from 12 independent experiments in each group and were analyzed statistically with one-way or two-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

SAHA Produced an Antiallodynia Effect in Rats Following Spinal Nerve Ligation

Paw mechanical withdrawal thresholds (PMWT) were measured 1 day before intrathecal catheter implantation (baseline value, BL), 1 day before neuropathic pain surgery (-1 day), and 1–28 days (day 1, 3, 5, 7, 14, 21, 28) after neuropathic pain surgery. According to a previous description, HDACIs treatment attenuates mechanical or thermal hypersensitivity only when used before any injury [10]. Our results showed that SNL caused a dramatic decrease in the PMWT 1 day after surgery (PMWT: naïve vs v-SNL, $P < 0.001$, Fig. 1). The PMWT reduced to the lowest value on the third day after surgery and then remained constant for at least 28 days, as previously described [21]. There were no PMWT changes in the naïve rats and all the sham group rats (Fig. 1). However, as expected, SAHA significantly reduced the duration of mechanical allodynia and increased the PMWT on the third day after surgery (PMWT: l-SNL versus v-SNL, $P < 0.001$; h-SNL versus v-SNL, $P < 0.001$, Fig. 1), as measured by von Frey filaments compared with the v-SNL group. Furthermore, this effect of SAHA in ameliorating mechanical allodynia persisted throughout the entire experiment, while no significant difference was found between the l-SNL group and the h-SNL group. Therefore, our results showed that intrathecal administration of SAHA had an antinociceptive effect on neuropathic pain induced by SNL.

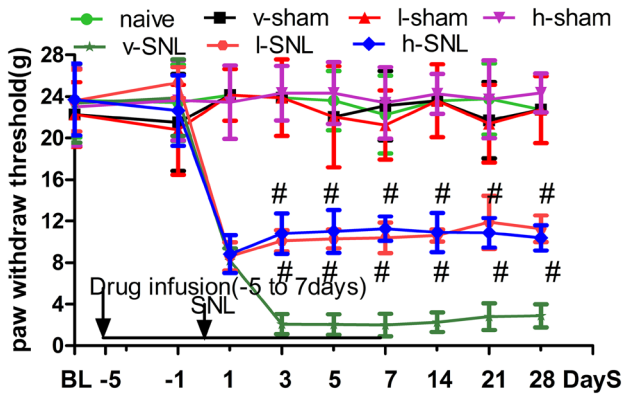


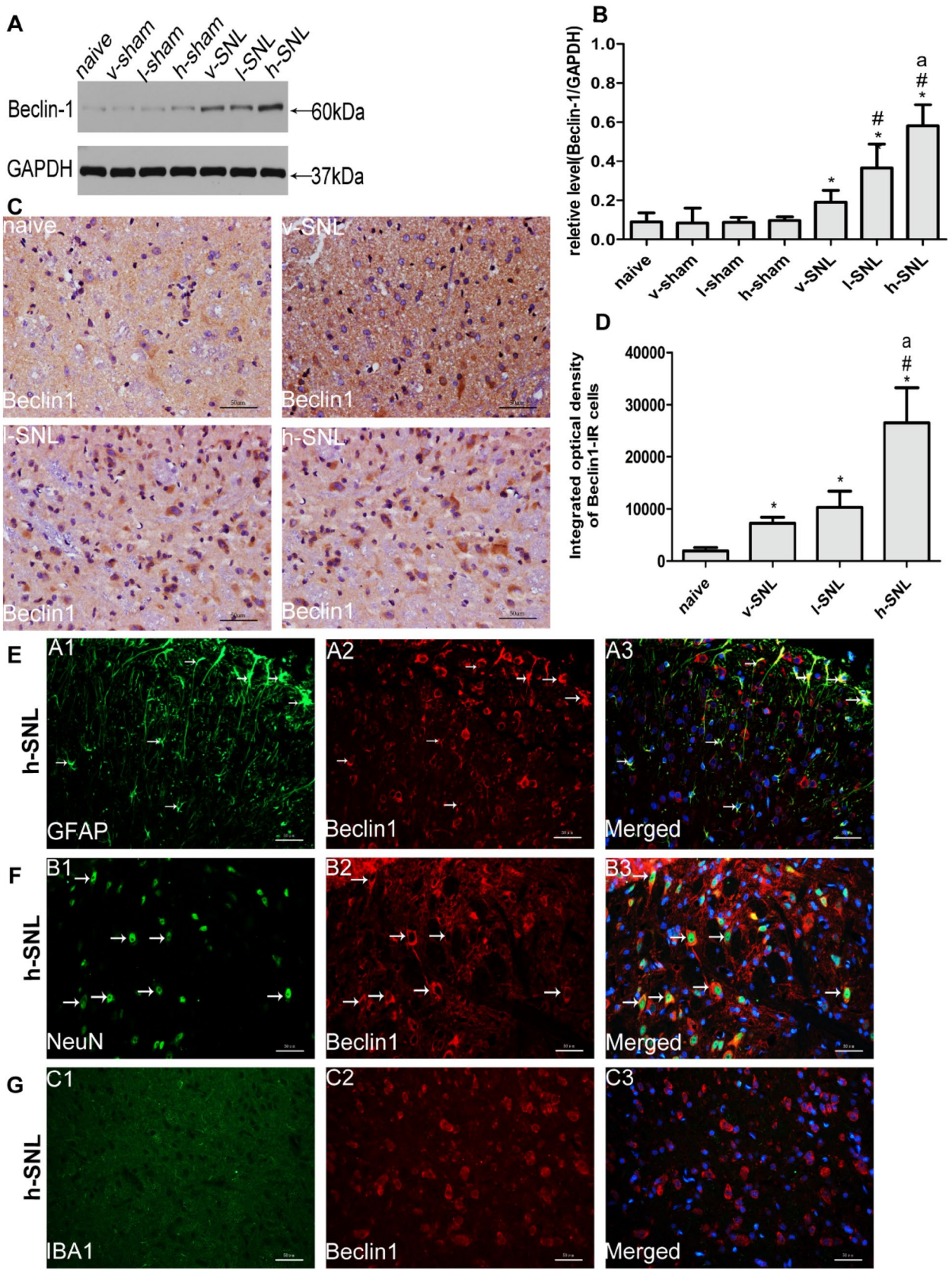
Fig. 1 The paw mechanical withdrawal threshold (PMWT) tests were carried out before catheter implantation (baseline value, BL), one day before spinal nerve ligation surgery (-1), and on days 1, 3, 5, 7, 14, 21, and 28 postsurgery. SAHA was intrathecally injected for 12 days starting 5 days before SNL and continued until 7 days after surgery. Each test was repeated 3 times on the same paw in 5-min intervals to obtain the 50% pain threshold. Data are presented as the mean ± standard deviation (SD) of 12 rats for each group, l-SNL group and h-SNL group compared with the v-SNL group, $^{\#}P < 0.05$; there were no significant differences between the naïve and all sham groups

Beclin-1 Expression in the Spinal Dorsal Horn Following SAHA Treatment

Recent evidence has suggested that Beclin-1 and PIK3C3/VPS34 are essential partners in the autophagy interactome that signals the onset of autophagy [26]. To further investigate whether autophagy induction increased after SAHA treatment, we detected the expression of Beclin-1 by western blot and immunohistochemical analysis on day 28 after SNL surgery. Western blot analysis showed that SNL induced a statistically significant increase in Beclin-1 expression in the lumber spinal dorsal horn compared with the control groups (Beclin-1: naïve vs. v-SNL, $P = 0.026$; different sham goups vs. v-SNL, $P < 0.05$, Fig. 2a, b). Our results showed that SAHA significantly increased Beclin-1 expression (Beclin-1: v-SNL vs. l-SNL, $P < 0.00$, Fig. 2a, b) and SAHA showed a dose-dependent effect on regulating Beclin-1 expression (Beclin-1: h-SNL vs. l-SNL, $P < 0.00$, Fig. 2a, b). Immunohistochemical analysis also showed that the high dose of SAHA significantly contributed to Beclin-1 expression (Fig. 2c, d), although, no significant difference was observed between the v-SNL and l-SNL group. Double immunofluorescent histochemistry staining was performed to detect the cellular localization of Beclin-1 expression in the spinal dorsal horn, which showed that Beclin-1 was mostly expressed in astrocytes and neuronal cells (Fig. 2e, f). Hence, SAHA contributed to the expression of Beclin-1 in spinal astrocytes and neuronal cells of SNL rats and had a dose dependent effect.

LC3 Levels in Spinal Dorsal Horn Following SAHA Treatment

In addition to Beclin-1, the microtubule associated protein light chain 3 (LC3) is also widely used as a marker of autophagy. In mammalian cells, LC3 has nonlipidated and lipidated forms, which are usually referred to as LC3I and LC3II, respectively [26]. LC3II has already been considered a reliable autophagy-related protein marker, which indicates autophagosome formation. The conversion of LC3I to LC3II is increased or the ratio of LC3II to LC3I is decreased when autophagy is induced [26]. We also detected the expression of LC3I and LC3II in the spinal dorsal horn after behavioral tests. Western blot analysis showed that SNL induced upregulation of LC3II and increased the ratio of LC3II to LC3I compared with the control groups (LC3II: naïve vs. v-SNL, $P < 0.00$; sham groups vs. v-SNL, $P < 0.00$; LC3II/LC3I: naïve vs. v-SNL, $P < 0.00$; sham groups vs. v-SNL, $P < 0.00$, Fig. 3a, b). Interestingly, SAHA further increased LC3II expression (LC3II: l-SNL vs. v-SNL, $P < 0.00$;


A
naive
v-sham
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h-sham
v-SNL
l-SNL
h-SNL
Beclin-1
60kDa
GAPDH
37kDa
B
reletive level(Beclin-1/GAPDH)
1.0
0.8
0.6
0.4
0.2
0.0
naive
v-sham
l-sham
h-sham
v-SNL
l-SNL
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naive
v-SNL
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D
Integrated optical density of Beclin1-IR cells
40000
30000
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10000
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E
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A1
A2
A3
GFAP
Beclin1
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B2
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NeuN
Beclin1
Merged
G
h-SNL
C1
C2
C3
IBA1
Beclin1
Merged

◂**Fig. 2** SAHA treatment induces a change in Beclin-1 modulation in neuropathic pain rats with spinal nerve ligation. **a** Immunoblotting showing the level of Beclin-1 in the ipsilateral spinal dorsal horn in the naïve group, v-sham group, l-sham group, h-sham group, v-SNL group, l-SNL group and h-SNL group (n = 6 in each group). **b** A higher intrathecal dose of SAHA significantly increased the Beclin-1 level compared with the v-SNL group and the l-SNL group. **c** Beclin-1 expression (DAB staining) in the spinal dorsal horn was detected by immunohistochemistry (scale bars = 50 μm, n = 3 in each group and 6 slices per rats were employed). **d** The integral optical density (IOD SUM) of Beclin-1 immune reactive (IR) cells in the ipsilateral spinal dorsal horn was determined by immunohistochemistry, and measured by Image-Pro Plus. A higher intrathecal dose of SAHA significantly increased the Beclin-1 level compared with the v-SNL group and the l-SNL group. **e** Double immunostaining showed that Beclin-1 (red) was present in astrocytes (arrows in A1–A3) and neuronal cells (B1–B3), as shown by the GFAP and NeuN (green)-positive structure in the h-SNLgroup. Scale bars = 50 μm, n = 3 in each group and 6 slices per rats were employed. Compared with control groups, * $P < 0.05$; compared with the v-SNL group, $^{\#}P < 0.05$; compared with the l-SNL group, $^{a}P < 0.05$

h-SNL vs. v-SNL, $P < 0.01$; LC3II/ LC3I: l-SNL vs. v-SNL, $P < 0.00$; h-SNL vs. v-SNL, $P < 0.00$, Fig. 3a, b). Furthermore, compared with the l-SNL group, there was a further increase in LC3II expression in the h-SNL group (LC3II: h-SNL vs. l-SNL, $P = 0.01$; LC3II/LC3: h-SNL vs. l-SNL, $P = 0.003$, Fig. 3a, b). The immunohistochemical analysis revealed a similar result (Fig. 3c, d). Double immunofluorescence staining also showed that LC3II was mostly expressed in astrocytes and neuronal cells in the spinal dorsal horn (Fig. 3e, f). These data suggest that SAHA contributes to LC3II expression in astrocytes and neuronal cells of the spinal dorsal horn after SNL.

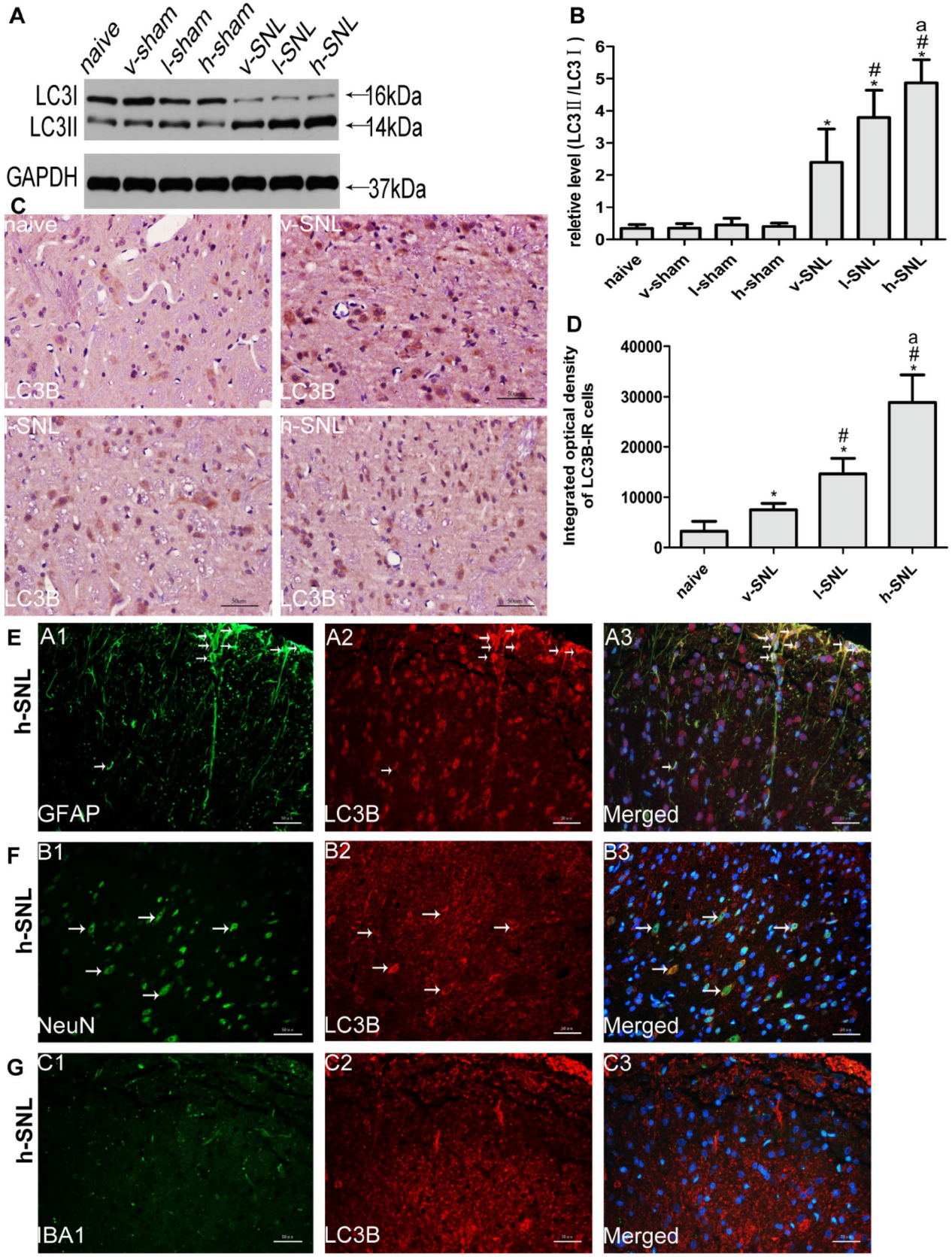
SAHA Enhances Autophagy Activity in the Spinal Dorsal Horn

Upregulation of LC3II can result from upregulated levels of autophagy but also from damaged autophagy flux. P62 is degraded in autolysosomes via binding to LC3 [26, 44], serving as an index of autophagy degradation. Therefore, we investigated the expression of P62/SQSTM1. In the v-SNL group, P62 was significantly upregulated in the lumber spinal dorsal horn compared with these control groups (P62: naïve vs. v-SNL, $P < 0.00$; sham groups vs. v-SNL, $P < 0.00$, Fig. 4a, b). In contrast, SAHA treatment reversed this effect (P62: l-SNL vs. v-SNL, $P < 0.00$; h-SNL vs. v-SNL, $P < 0.00$, Fig. 4a, b). The immunohistochemical analysis exhibited the same result, as we also found that a higher dose of SAHA had a stronger effect in reducing P62 expression (Fig. 4c, d). Furthermore, we found that SAHA contributed to autophagic contents degradation, as we mainly detected a higher capacity to degrade autophagic contents under TEM compared with the v-SNL group (Fig. 4e, f). Thus, these results including the expressions of LC3, Beclin-1 and P62, revealed that SNL disrupted autophagy in ipsilateral lumber spinal dorsal horn astrocytes and resulted in the development and maintenance of pain, while SAHA reversed this effect. Moreover, the effect of SAHA seemed dose related, as a higher dose of SAHA had a stronger effect on regulating autophagy flux on the spinal astrocytes of the SNL rats. Hence, SAHA induces lysosome-autophagosome fusion, contributes to degradation of the spinal astrocytes of SNL rats, and ameliorates neuropathic pain.

mTOR and Phospho-mTOR Expression in the Spinal Dorsal Horn of SNL Rats were Decreased upon SAHA Treatment

Several lines of evidence have suggested that SAHA can stimulate autophagy by regulating mTOR under some pathological conditions. To further test whether SAHA treatment inhibits the mTOR pathway and increases autophagy in the spinal dorsal horn, we then detected the effects of intrathecally administrating rapamycin on mTOR and its activated form, phosphorylated mTOR (p-mTOR), in the spinal dorsal horn. Behavioral tests showed that rapamycin significantly increased the PMWT of SNL rats (PMWT: r-SNL vs. v-SNL, $P < 0.00$, Fig. 5a); however, there were no significant differences in the antinociceptive effect between the r-SNL group and the h-SNL group ($P > 0.05$, Fig. 5a).

Western blot analysis showed that mTOR showed a slightly upregulated expression after SNL surgery (mTOR: v-SNL vs. r-sham, $P < 0.00$; r-SNL vs r-sham, $P = 0.001$; h-SNL vs. r-sham, $P = 0.002$, Fig. 5b, d), but there was no difference between the v-SNL, r-SNL and h-SNL groups. Moreover, p-mTOR showed significantly increased expression after SNL surgery (p-mTOR: v-SNL vs. r-sham, $P < 0.00$; r-SNL vs. r-sham, $P = 0.378$; h-SNL vs. r-sham, $P = 0.49$, Fig. 5c, e), and this effect could be suppressed by intrathecal injection of SAHA or rapamycin (p-mTOR: h-SNL vs. v-SNL, $P < 0.00$; r-SNL vs. v-SNL, $P < 0.00$ Fig. 5c, e). We did not observe a significant difference in p-mTOR expression between the r-SNL group and the h-SNL group ($P = 0.779$). Interestingly, according to the double immunofluorescence staining analysis, p-mTOR, GFAP and NeuN fluorescence staining were observed to be colocalized (Fig. 5f, g). Taken together, these data suggest a sustained reduction in mTOR and p-mTOR, mainly p-mTOR induced by SAHA. Hence, the amelioration of neuropathic pain by SAHA observed in this study was associated with upregulated autophagy flux via inhibiting the mTOR and p-mTOR pathways in the spinal dorsal horn, especially in astrocytes and neuronal cells.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l-sham
h-sham
v-SNL
l-SNL
h-SNL
LC3I
LC3II
16kDa
14kDa
GAPDH
37kDa
B
relative level (LC3 II /LC3 I)
naive
v-sham
l-sham
h-sham
v-SNL
l-SNL
h-SNL
C
naive
v-SNL
l-SNL
h-SNL
LC3B
D
Integrated optical density of LC3B-IR cells
E
h-SNL
A1
GFAP
A2
LC3B
A3
Merged
F
B1
NeuN
B2
B3
G
C1
IBA1
C2
C3

◂**Fig. 3** SAHA treatment induces a change in LC3 modulation in neuropathic pain rats with spinal nerve ligation. **a** Immunoblotting showing the levels of LC3I and LC3II in the ipsilateral spinal dorsal horn in these seven groups (n = 6 in each group). **b** A higher intrathecal dose of SAHA significantly increased the LC3II level compared with the v-SNL group and the l-SNL group. **d** LC3 expression (DAB staining) in the ipsilateral spinal dorsal horn was detected by immunohistochemistry (scale bars = 50 μm, n = 3 in each group and 6 slices per rats were employed). **e** The integral optical density (IOD SUM) of LC3B immune reactive (IR) cells in the ipsilateral spinal dorsal horn was determined by immunohistochemistry, as measured by Image-Pro Plus. A higher intrathecal dose of SAHA significantly increased the LC3B level compared with the v-SNL group or the l-SNL group. **f** Double immunostaining showed that LC3B (red) was present in astrocytes (arrows in A1–A3) and neuronal cells (arrows in B1–B3), as shown by the GFAP (green)-positive structure in the h-SNLgroup. Scale bars = 50 μm, n = 3 in each group and 6 slices per rats were employed. Compared with control groups, $^{*}P < 0.05$; compared with the v-SNL group, $^{\#}P < 0.05$; compared with the l-SNL group, $^{a}P < 0.05$

Discussion

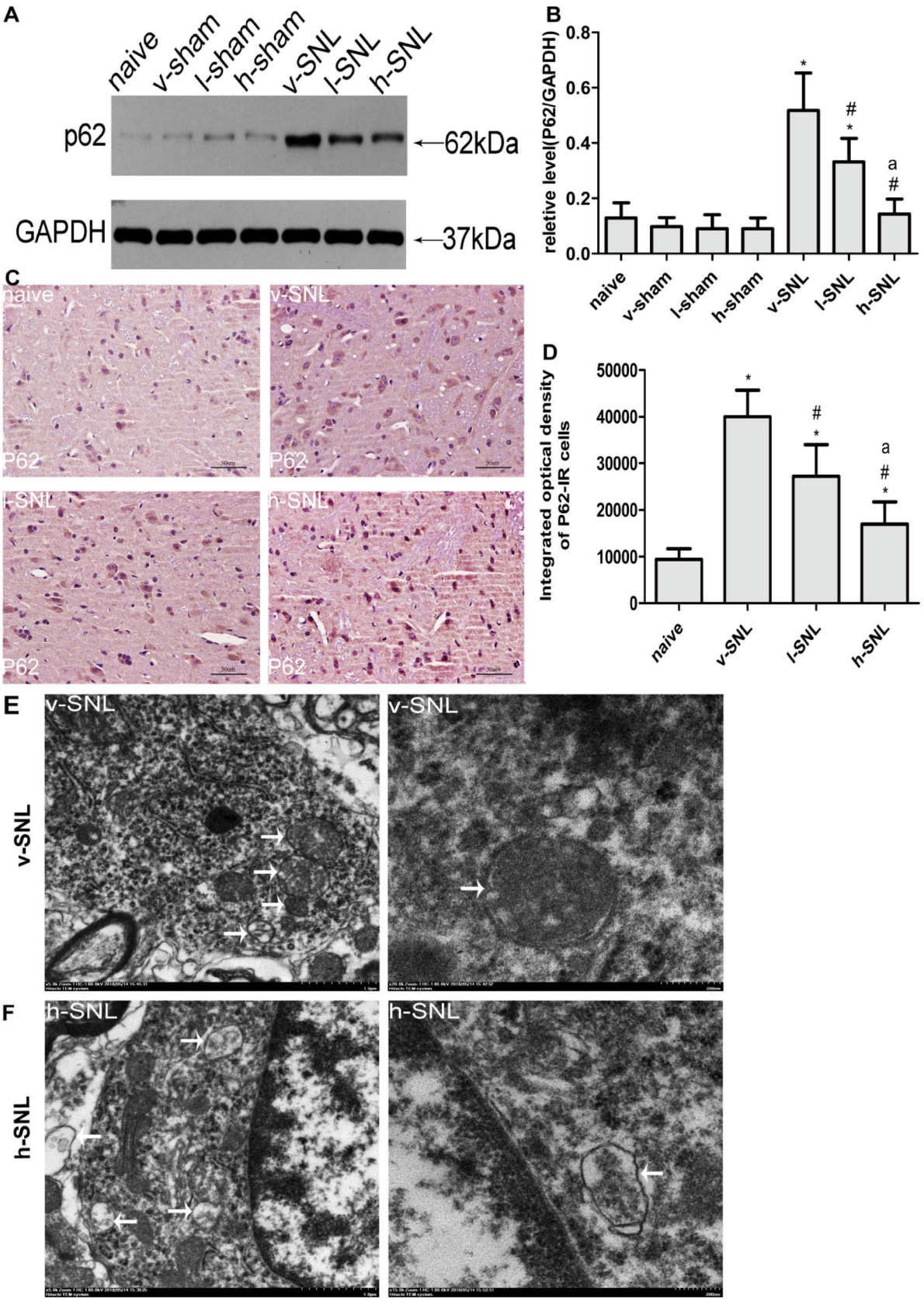
Neuropathic pain is widely considered one of the most intractable pain conditions to manage, with unsatisfactory clinical outcomes, and often leads to chronic pain situations [45]. Chronic pain seriously affects the patients' physical and mental health, greatly reduces the quality of life and significantly increases the economic burden of their family. The roles of autophagy in the progression of chronic pain pathology have been widely researched [18], which is still an urgent issue that needs to be solved. The peripheral nerve injury model produces chronic pain hypersensitivities in rodent animal models that mimic clinical chronic neuropathic pain.

To date, many studies have focused on neuronal or microglial autophagy, while few have investigated the role of astrocyte autophagy in neuropathic pain pathology. It has been extensively researched and accepted that astrocytes result in the maintenance of neuropathic pain [35, 37]. The key finding of our study is that an intrathecal dose of SAHA is sufficient to induce autophagy, accelerate its contents degradation and ameliorate pain via suppressing the mTOR pathway in male neuropathic pain rats. This effect also showed a dose-dependent effect. This conclusion is supported by an increase in the PMWT by von Frey filaments and an increase in the expression of autophagy markers according to western blot analysis and immunofluorescence staining after intrathecal injection of SAHA. SNL rats that received SAHA treatment displayed decreased mTOR or p-mTOR (especially p-mTOR) expression in the spinal dorsal horn, which also contributes to our findings. In addition, we tentatively suggest that intrathecal injection of 200 nmol SAHA once a day might be a proper dose for ameliorating neuropathic pain, as this dose showed a better effect on regulating autophagy and p-mTOR expression. Our study provides a promising start to further investigate new treatments for neuropathic pain.

Several experimental studies have documented that peripheral nerve injury increases the levels of histone deacetylase (HDAC), resulting in a significant decrease in histone acetylation, which is correlated with the induction of neuropathic pain. Accordingly, HDACIs have been shown to ameliorate pain in models of neuropathic pain [9, 10]. In fact, the administration of HDACIs improved several symptoms of neuropathic pain. In addition to cancer treatment, SAHA has recently been used therapeutically in neuropathic pain. Importantly, an intrathecal dose of SAHA was found to efficiently reduce spinal nerve ligation-induced hyperalgesia and mechanical allodynia [13, 46]. We found that a higher dose of SAHA treatment showed antiallodynia effects, which is consistent with previous studies showing that HDACIs attenuated neuropathic pain and inflammatory pain [10, 11, 46]. However, no significant differences in PMWT were measured between rats treated with 100 nmol/day and 200 nmol/day SAHA, which may be related to the different measurement tools used, the various experimental models, and so on.

Autophagy is a major intracytoplasmic protein degradation pathway. The first morphological characteristic structure in autophagy is the double-membrane, called the phagophore, that engulfs substrates as it matures into complete autophagosomes, which are stilled bordered by a double membrane. Finally, it traffics along microtubules to enable autophagosome–lysosome fusion, which leads to the degradation of autophagic contents [26]; this process is known as autophagic flux. Autophagy in the spinal dorsal horn plays a critical role in alleviating neuropathic pain following peripheral nerve injury [21, 22]. Many studies have revealed that autophagic flux is disrupted in the development of neuropathic pain [21, 24], but the cell type or phase of autophagy disruption in the spinal dorsal horn remains unclear.

Beclin-1 and PIK3C3/VPS34 are essential partners in the autophagy interactome that signal the onset of autophagy [26]. In our study, Beclin-1 expression levels were significantly increased in the lumber spinal dorsal horn after SNL surgery. Beclin-1 expression levels were significantly accumulated in the astrocyte cells and neuronal cells after SAHA treatment. Studies have revealed that a common complex composed of Beclin-1 and ULK1 is a key requirement for autophagy induction [47]. Thus, Beclin-1 accumulation may indicate an increase in autophagy flux or disabled autophagosome degradation. Thus, we further investigated the expression of other autophagy-related proteins. LC3II, which is known to be associated with autophagosomes, is a reliable autophagy-related protein marker. Significant increases in the expression of LC3II and LC3II relative to LC3I were observed in the SNL group. Moreover, SAHA further increased the expression of LC3II, without a


A
naive
v-sham
l-sham
h-sham
v-SNL
l-SNL
h-SNL
p62
62kDa
GAPDH
37kDa
B
reletive level(P62/GAPDH)
0.8
0.6
0.4
0.2
0.0
naive
v-sham
l-sham
h-sham
v-SNL
l-SNL
h-SNL
C
naive
P62
v-SNL
P62
l-SNL
P62
h-SNL
P62
D
Integrated optical density of P62-IR cells
50000
40000
30000
20000
10000
0
naive
v-SNL
l-SNL
h-SNL
E
v-SNL
v-SNL
v-SNL
F
h-SNL
h-SNL
h-SNL

◂**Fig. 4** SAHA treatment induces a change in P62 modulation in neuropathic pain rats with spinal nerve ligation. **a** Immunoblotting shows the level of P62 in the ipsilateral spinal dorsal horn in the different groups above (n = 6 in each group). **b** A higher intrathecal dose of SAHA significantly reduced the P62 level compared with the v-SNL group and the l-SNL group. **c** P62 expression (DAB staining) in the ipsilateral spinal dorsal horn was detected by immunohistochemistry (scale bars = 50 μm, n = 3 in each group and 6 slices per rats were employed). **d** The integral optical density (IOD SUM) of P62 immune reactive (IR) cells in the ipsilateral spinal dorsal horn was determined by immunohistochemistry, measured by Image-Pro Plus. A higher intrathecal dose of SAHA significantly reduced the P62 level vs the v-SNL group or the l-SNL group. **e, f** Transmission electron microscopy (TEM) showed that SAHA had a higher capacity to degrade autophagic contents in the h-SNL group than that in the v-SNL group (A2, B2 scale bars = 1 μm; A3, C3 scale bars = 500 nm; n = 3 in each group and 6 slices per rats were employed). Compared with control groups, $^{*}P < 0.05$; compared with the v-SNL group, $^{\#}P < 0.05$; compared with the l-SNL group, $^{a}P < 0.05$

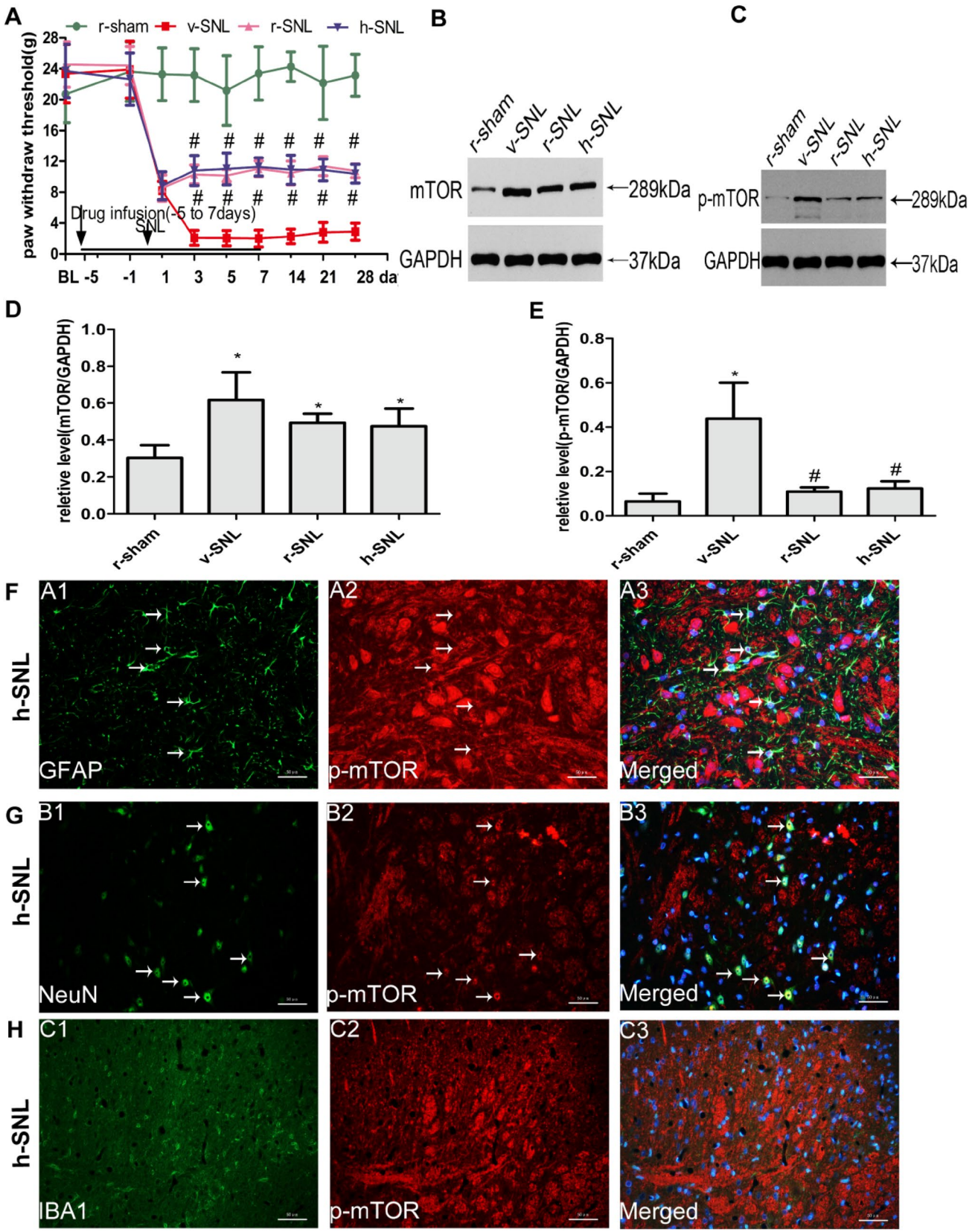
significant change in LC3I. LC3II accumulation can result from increased formation of autophagosomes/autophagolysosomes but also from impairment at one of the last steps, such as fusion with lysosomes or cargo degradation [48]. Therefore, it is preferable to integrate LC3 studies with the analysis of other components of autophagic machinery, such as members of the initiation complex (i.e., Beclin-1) or autolysosome substrates (i.e., SQSTM1/P62) [26].

P62/SQSTM1 levels are upregulated when autophagy is impaired [44]. Our results showed that P62 levels were significantly increased in the lumber spinal dorsal horn of the SNL rats but not in the control rats. In contrast, SAHA dramatically decreased the expression of P62, which is an autophagic flux marker. Studies of SNL models of neuropathic pain have suggested a block in the late phases of autophagic flux rather than an induction of the process [21, 49]. In our research, LC3II and Beclin-1 levels were significantly increased, but P62 levels were significantly decreased in the SAHA-treated rats. Under TEM, we found that SAHA induced lysosome-autophagosome fusion and degradation, which also signaled that SAHA plays a role in contributing to autophagy flux. These results revealed that an intrathecal dose of SAHA could accelerate autophagy degradation in the ipsilateral lumber spinal dorsal horn, ameliorating neuropathic pain.

We further studied the role of autophagy in different type cells in the spinal dorsal horn as mediated by SAHA treatment in neuropathic pain. Astrocytes are the most prevalent cell type in the CNS. Astrocyte activation is the main mechanism in the maintenance of neuropathic pain [21]. Moreover, astrocytes are activated within 24 h of nerve injury or inflammation, which is later than microglia, but remain functional for up to 12 weeks [37, 50]. However, Echeverry et al. [36] revealed that long-lasting spinal microglia activation contributes to the maintenance of neuropathic pain in the chronic phase. In the present study, we found that LC3, Beclin-1, and P62 expression associated with autophagy impairment in SNL-induced neuropathic pain rats following SAHA treatment was mainly localized with astrocytes and neuronal cells, rather than microglia cells in the ipsilateral lumber spinal dorsal horn. These observations included increased levels of Beclin-1 and LC3II or decreased levels of P62 following SAHA treatment, suggesting that SAHA attenuated neuropathic pain by upregulating autophagy flux. These facts suggest that intrathecal administration of SAHA mainly contributes to astrocyte and neuronal cells autophagy flux and reduces neuropathic pain conditions. SNL caused disruption of autophagy and then resulted in the development and maintenance of pain. As expected, SAHA reversed this effect. Moreover, the effect of SAHA appeared to be dose related, as a higher dose of SAHA (n = 200 nmol) had a stronger effect on regulating autophagy flux. Therefore, it is reasonable to suggest that SAHA increases autophagy flux in astrocytes and neuronal cells, reducing pain sensory hypersensitivity.

Recent studies have suggested that SAHA induces autophagy by inhibiting the mTOR pathway. Therefore, we hypothesized that SAHA may also regulate the mechanism of neuropathic pain, and we explored spinal dorsal horn modulation of several autophagy markers (LC3, Beclin-1, P62) in rats after all behavioral tests. On the other hand, we found that SAHA increased the conversion of LC3II and the expression of Beclin-1 and decreased the expression of P62/SQSTM1, which are molecular markers of autophagy. On the other hand, we found that SAHA induced autophagic content degradation by TEM.

Systemic or local administration of rapamycin, a specific inhibitor of mTOR, has been shown to alleviate mechanical hypersensitivity induced by peripheral nerve injury [30, 51]. mTOR and AMP-responsive protein kinase (AMPK) are the main autophagy regulators. mTOR plays a basic role in the initiation and maturation of autophagy by controlling signal transduction cascades involved in this process [52]. It has been recently reported that SAHA triggers autophagy through downregulation of the mTOR pathway [25, 27]. The mTOR pathway has also been shown to participate in regulating central and peripheral nociceptive sensitization [34]. However, whether mTOR and phosphor-mTOR (p-mTOR) are activated in the spinal cord after peripheral nerve injury is still controversial. Our study further clarifies the correlation between SAHA and autophagy in neuropathic pain. We hypothesized that SAHA might regulate autophagy and contribute to nociceptive sensitization in a rat model of neuropathic pain. Geranton and his colleagues found no changeable expression of p-mTOR or its downstream effectors (P-S6K1) in the superficial dorsal horn 7 days after spared nerve injury [53]. Asante et al. [33] showed that SNL reduced the expression of P-S6K1 in the superficial dorsal horn


A
r-sham
v-SNL
r-SNL
h-SNL
paw withdraw threshold(g)
Drug infusion(-5 to 7days)
SNL
BL -5 -1 1 3 5 7 14 21 28 da
B
r-sham v-SNL r-SNL h-SNL
mTOR
289kDa
GAPDH
37kDa
C
r-sham v-SNL r-SNL h-SNL
p-mTOR
289kDa
GAPDH
37kDa
D
reletive level(mTOR/GAPDH)
r-sham v-SNL r-SNL h-SNL
E
reletive level(p-mTOR/GAPDH)
r-sham v-SNL r-SNL h-SNL
F
h-SNL
A1
GFAP
A2
p-mTOR
A3
Merged
G
h-SNL
B1
NeuN
B2
p-mTOR
B3
Merged
H
h-SNL
C1
IBA1
C2
p-mTOR
C3
Merged

◂**Fig. 5** Effects of the administration of SAHA (200 nmol) and rapamycin on mechanical allodynia and mTOR or p-mTOR expression in astrocytes of SNL rats. **a** SAHA or rapamycin was intrathecally injected for 12 days starting 5 days before SNL and until 7 days after surgery. Intrathecal doses of SAHA or rapamycin significantly increased the PMWT ($^{\#}P<0.05$ compared with v-SNL group; $^{*}P<0.05$ compared with the r-sham group, n = 12 in each group). **b, c** SAHA or rapamycin treatment induces a change in mTOR or p-mTOR expression in neuropathic pain rats with spinal nerve ligation. **d, e** Immunoblotting showing the level of mTOR or p-mTOR in the ipsilateral spinal dorsal horn in the r-sham group, v-SNL group, r-SNL group and h-SNL group (n = 6 in each group). A higher intrathecal dose of SAHA significantly reduced p-mTOR levels. **e** Double immunostaining showed that p-mTOR (red) was present in astrocyte and neuronal cells, as shown by the GFAP and NeuN (green)-positive structure in the h-SNL group. Scale bars = 50 μm, n = 3 in each group, and 6 slices per rats were employed. Compared with control groups, $^{*}P<0.05$; compared with the v-SNL group, $^{\#}P<0.05$; compared with the l-SNL group, $^{a}P<0.05$

14 days after surgery. Lingli et al. [54] then revealed that SNL did not alter the expression of either p-mTOR or mTOR in the ipsilateral L5 spinal cord 14 days after surgery. Recently, Tillu et al. [34] showed that CCI induced the expression of mTOR in activated astrocytes on day 14 after SNL operation. Intrathecal injection of SAHA in SNL rats undergoing nerve injury increased pain hypersensitivity. It seems that SAHA mainly regulates p-mTOR expression in astrocytes and neuronal cells, as we observed that SNL upregulated the levels of p-mTOR and mTOR in the ipsilateral L5 spinal cord 4 weeks after surgery. Moreover, intrathecal administration of SAHA significantly reduced p-mTOR expression, rather than mTOR expression in our study. Double immunofluorescent staining showed that p-mTOR was mainly expressed in spinal astrocytes and neuronal cells. These data are consistent with our hypothesis of mTOR inhibition in SAHA-treated SNL rats. We found that SAHA treatment did not abolish pain hypersensitivity. There may be other pathways involved in pain regulation, and this area needs future research.

SAHA nonspecifically inhibits HDACs, leading to an accumulation of acetylated histones H2a, H2b, H3 and H4 [55, 56]. Studies have shown that upregulated HDAC1 expression and reduced histone H3 acetylation play vital roles in the development and maintenance of neuropathic pain [57]. HDAC inhibitors attenuate the development of hypersensitivity in models of neuropathic pain after different class I HDAC inhibitors are delivered intrathecally to the spinal cord [10, 13, 58]. In addition, histone modifications are implicated in the autophagic process. Therefore, in the last few years, HDAC inhibitors have become a research focus in neuropathic pain therapy. In our system, we did not further explore the expression of HDACs. In future studies, the correlation between HDACs and autophagy in pain models of neuropathic pain needs to be further elucidated.

Conclusion

In the present study, we indicated that the degradation of autophagy (the late phase of autophagy flux) was blocked in an SNL model of neuropathic pain. An intrathecal dose of SAHA is sufficient to ameliorate pain by suppressing p-mTOR expression in male neuropathic pain rats.

Acknowledgements This work was supported by the National Nature Science Foundation of China (No. 81471858) and the Scientific Research Program of Hubei Health and Family Planning Committee (No. WJ2017M036).

Compliance with Ethical Standards

Conflict of interest There are no conflicts of interest.

References

1. Baron R (2006) Mechanisms of disease: neuropathic pain—a clinical perspective. Nat Clin Pract Neurol 2(2):95–106. https://doi.org/10.1038/ncpneuro0113
2. Dworkin RH, Backonja M, Rowbotham MC, Allen RR, Argoff CR, Bennett GJ, Bushnell MC, Farrar JT, Galer BS, Haythornthwaite JA, Hewitt DJ, Loeser JD, Max MB, Saltarelli M, Schmader KE, Stein C, Thompson D, Turk DC, Wallace MS, Watkins LR, Weinstein SM (2003) Advances in neuropathic pain: diagnosis, mechanisms, and treatment recommendations. Arch Neurol 60(11):1524–1534. https://doi.org/10.1001/archneur.60.11.1524
3. Baron R (2009) Neuropathic pain: a clinical perspective. Handb Exp Pharmacol. https://doi.org/10.1007/978-3-540-79090-7_1
4. Jensen TS, Baron R, Haanpaa M, Kalso E, Loeser JD, Rice AS, Treede RD (2011) A new definition of neuropathic pain. Pain 152(10):2204–2205. https://doi.org/10.1016/j.pain.2011.06.017
5. Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, Freeman R, Truini A, Attal N, Finnerup NB, Eccleston C, Kalso E, Bennett DL, Dworkin RH, Raja SN (2017) Neuropathic pain. Nat Rev Dis Prim 3:17002. https://doi.org/10.1038/nrdp.2017.2
6. Fiore NT, Austin PJ (2016) Are the emergence of affective disturbances in neuropathic pain states contingent on supraspinal neuroinflammation? Brain Behav Immun 56:397–411. https://doi.org/10.1016/j.bbi.2016.04.012
7. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, Gilron I, Haanpaa M, Hansson P, Jensen TS, Kamerman PR, Lund K, Moore A, Raja SN, Rice AS, Rowbotham M, Sena E, Siddall P, Smith BH, Wallace M (2015) Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol 14(2):162–173. https://doi.org/10.1016/s1474-4422(14)70251-0
8. Hearn L, Derry S, Phillips T, Moore RA, Wiffen PJ (2014) Imipramine for neuropathic pain in adults. Cochrane Database Syst Rev. https://doi.org/10.1002/14651858.CD010769.pub2
9. Uchida H, Matsushita Y, Araki K, Mukae T, Ueda H (2015) Histone deacetylase inhibitors relieve morphine resistance in neuropathic pain after peripheral nerve injury. J Pharmacol Sci 128(4):208–211. https://doi.org/10.1016/j.jphs.2015.07.040
10. Denk F, Huang W, Sidders B, Bithell A, Crow M, Grist J, Sharma S, Ziemek D, Rice AS, Buckley NJ, McMahon SB (2013) HDAC inhibitors attenuate the development

of hypersensitivity in models of neuropathic pain. Pain 154(9):1668–1679. https://doi.org/10.1016/j.pain.2013.05.021
11. Bai G, Wei D, Zou S, Ren K, Dubner R (2010) Inhibition of class II histone deacetylases in the spinal cord attenuates inflammatory hyperalgesia. Mol Pain 6:51. https://doi.org/10.1186/1744-8069-6-51
12. Denk F, McMahon SB (2012) Chronic pain: emerging evidence for the involvement of epigenetics. Neuron 73(3):435–444. https://doi.org/10.1016/j.neuron.2012.01.012
13. Matsushita Y, Araki K, Omotuyi O, Mukae T, Ueda H (2013) HDAC inhibitors restore C-fibre sensitivity in experimental neuropathic pain model. Br J Pharmacol 170(5):991–998. https://doi.org/10.1111/bph.12366
14. Liang L, Lutz BM, Bekker A, Tao YX (2015) Epigenetic regulation of chronic pain. Epigenomics 7(2):235–245. https://doi.org/10.2217/epi.14.75
15. Kukkar A, Singh N, Jaggi AS (2014) Attenuation of neuropathic pain by sodium butyrate in an experimental model of chronic constriction injury in rats. J Formos Med Assoc 113(12):921–928. https://doi.org/10.1016/j.jfma.2013.05.013
16. Xu K, Dai XL, Huang HC, Jiang ZF (2011) Targeting HDACs: a promising therapy for Alzheimer's disease. Oxidative Med Cell Longev 2011:143269. https://doi.org/10.1155/2011/143269
17. Klionsky DJ (2007) Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol 8(11):931–937. https://doi.org/10.1038/nrm2245
18. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ (2007) Potential therapeutic applications of autophagy. Nat Rev Drug Discov 6(4):304–312. https://doi.org/10.1038/nrd2272
19. Raudino F (2013) Non-cognitive symptoms and related conditions in the Alzheimer's disease: a literature review. Neurol Sci 34(8):1275–1282. https://doi.org/10.1007/s10072-013-1424-7
20. Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. Cell 132(1):27–42. https://doi.org/10.1016/j.cell.2007.12.018
21. Berliocchi L, Russo R, Maiaru M, Levato A, Bagetta G, Corasaniti MT (2011) Autophagy impairment in a mouse model of neuropathic pain. Mol Pain 7:83. https://doi.org/10.1186/1744-8069-7-83
22. Piao Y, Gwon DH, Kang DW, Hwang TW, Shin N, Kwon HH, Shin HJ, Yin Y, Kim JJ, Hong J, Kim HW, Kim Y, Kim SR, Oh SH, Kim DW (2018) TLR4-mediated autophagic impairment contributes to neuropathic pain in chronic constriction injury mice. Mol Barin 11(1):11. https://doi.org/10.1186/s13041-018-0354-y
23. Marinelli S, Nazio F, Tinari A, Ciarlo L, D'Amelio M, Pieroni L, Vacca V, Urbani A, Cecconi F, Malorni W, Pavone F (2014) Schwann cell autophagy counteracts the onset and chronification of neuropathic pain. Pain 155(1):93–107. https://doi.org/10.1016/j.pain.2013.09.013
24. Zhang E, Yi MH, Ko Y, Kim HW, Seo JH, Lee YH, Lee W, Kim DW (2013) Expression of LC3 and Beclin 1 in the spinal dorsal horn following spinal nerve ligation-induced neuropathic pain. Brain Res 1519:31–39. https://doi.org/10.1016/j.brainres.2013.04.055
25. Hrzenjak A, Kremser ML, Strohmeier B, Moinfar F, Zatloukal K, Denk H (2008) SAHA induces caspase-independent, autophagic cell death of endometrial stromal sarcoma cells by influencing the mTOR pathway. J Pathol 216(4):495–504. https://doi.org/10.1002/path.2434
26. Klionsky DJ, Abdelmohsen K, Abe A, Zughaier SM (2016) Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 12 (1):1–222. https://doi.org/10.1080/15548627.2015.1100356
27. Chiao MT, Cheng WY, Yang YC, Shen CC, Ko JL (2013) Suberoylanilide hydroxamic acid (SAHA) causes tumor growth slowdown and triggers autophagy in glioblastoma stem cells. Autophagy 9(10):1509–1526. https://doi.org/10.4161/auto.25664
28. Lee JY, Kuo CW, Tsai SL, Cheng SM, Chen SH, Chan HH, Lin CH, Lin KY, Li CF, Kanwar JR, Leung EY, Cheung CC, Huang WJ, Wang YC, Cheung CH (2016) Inhibition of HDAC3- and HDAC6-promoted survivin expression plays an important role in SAHA-induced autophagy and viability reduction in breast cancer cells. Front Pharmacol 7:81. https://doi.org/10.3389/fphar.2016.00081
29. Swiech L, Perycz M, Malik A, Jaworski J (2008) Role of mTOR in physiology and pathology of the nervous system. Biochim Biophys Acta 1784(1):116–132. https://doi.org/10.1016/j.bbapap.2007.08.015
30. Obara I, Tochiki KK, Geranton SM, Carr FB, Lumb BM, Liu Q, Hunt SP (2011) Systemic inhibition of the mammalian target of rapamycin (mTOR) pathway reduces neuropathic pain in mice. Pain 152(11):2582–2595. https://doi.org/10.1016/j.pain.2011.07.025
31. Obara I, Medrano MC, Signoret-Genest J, Jimenez-Diaz L, Geranton SM, Hunt SP (2015) Inhibition of the mammalian target of rapamycin complex 1 signaling pathway reduces itch behaviour in mice. Pain 156(8):1519–1529. https://doi.org/10.1097/j.pain.0000000000000197
32. Li G, Lu X, Zhang S, Zhou Q, Zhang L (2015) mTOR and Erk1/2 signaling in the cerebrospinal fluid-contacting nucleus is involved in neuropathic pain. Neurochem Res 40(5):1053–1062. https://doi.org/10.1007/s11064-015-1564-7
33. Asante CO, Wallace VC, Dickenson AH (2010) Mammalian target of rapamycin signaling in the spinal cord is required for neuronal plasticity and behavioral hypersensitivity associated with neuropathy in the rat. J Pain 11(12):1356–1367. https://doi.org/10.1016/j.jpain.2010.03.013
34. Tillu DV, Melemedjian OK, Asiedu MN, Qu N, De Felice M, Dussor G, Price TJ (2012) Resveratrol engages AMPK to attenuate ERK and mTOR signaling in sensory neurons and inhibits incision-induced acute and chronic pain. Mol Pain 8:5. https://doi.org/10.1186/1744-8069-8-5
35. Cao H, Zhang YQ (2008) Spinal glial activation contributes to pathological pain states. Neurosci Biobehav Rev 32(5):972–983. https://doi.org/10.1016/j.neubiorev.2008.03.009
36. Echeverry S, Shi XQ, Yang M, Huang H, Wu Y, Lorenzo LE, Perez-Sanchez J, Bonin RP, De Koninck Y, Zhang J (2017) Spinal microglia are required for long-term maintenance of neuropathic pain. Pain 158(9):1792–1801. https://doi.org/10.1097/j.pain.0000000000000982
37. Mika J, Zychowska M, Popiolek-Barczyk K, Rojewska E, Przewlocka B (2013) Importance of glial activation in neuropathic pain. Eur J Pharmacol 716(1–3):106–119. https://doi.org/10.1016/j.ejphar.2013.01.072
38. Lucocq JM, Hacker C (2013) Cutting a fine figure: on the use of thin sections in electron microscopy to quantify autophagy. Autophagy 9(9):1443–1448. https://doi.org/10.4161/auto.25570
39. Shih MH, Kao SC, Wang W, Yaster M, Tao YX (2012) Spinal cord NMDA receptor-mediated activation of mammalian target of rapamycin is required for the development and maintenance of bone cancer-induced pain hypersensitivities in rats. J Pain 13(4):338–349. https://doi.org/10.1016/j.jpain.2011.12.006
40. Sakura S, Kirihara Y, Muguruma T, Kishimoto T, Saito Y (2005) The comparative neurotoxicity of intrathecal lidocaine and bupivacaine in rats. Anesthesia Analg 101(2):541–547. https://doi.org/10.1213/01.ane.0000155960.61157.12 table of contents.
41. Norsted Gregory E, Codeluppi S, Gregory JA, Steinauer J, Svensson CI (2010) Mammalian target of rapamycin in spinal cord neurons mediates hypersensitivity induced by peripheral inflammation. Neuroscience 169(3):1392–1402. https://doi.org/10.1016/j.neuroscience.2010.05.067

42. Kim SH, Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50(3):355–363
43. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53(1):55–63
44. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol 171(4):603–614. https://doi.org/10.1083/jcb.200507002
45. Inoue S, Taguchi T, Yamashita T, Nakamura M, Ushida T (2017) The prevalence and impact of chronic neuropathic pain on daily and social life: a nationwide study in a Japanese population. Eur J Pain 21(4):727–737. https://doi.org/10.1002/ejp.977
46. Cui SS, Lu R, Zhang H, Wang W, Ke JJ (2016) Suberoylanilide hydroxamic acid prevents downregulation of spinal glutamate transporter-1 and attenuates spinal nerve ligation-induced neuropathic pain behavior. Neuroreport 27(6):427–434. https://doi.org/10.1097/wnr.0000000000000558
47. Antonioli M, Di Rienzo M, Piacentini M, Fimia GM (2017) Emerging mechanisms in initiating and terminating autophagy. Trends Biochem Sci 42(1):28–41. https://doi.org/10.1016/j.tibs.2016.09.008
48. Mizushima N, Yoshimori T, Levine B (2010) Methods in mammalian autophagy research. Cell 140(3):313–326. https://doi.org/10.1016/j.cell.2010.01.028
49. Berliocchi L, Maiaru M, Varano GP, Russo R, Corasaniti MT, Bagetta G, Tassorelli C (2015) Spinal autophagy is differently modulated in distinct mouse models of neuropathic pain. Mol Pain 11:3. https://doi.org/10.1186/1744-8069-11-3
50. Thakur KK, Saini J, Mahajan K, Singh D, Jayswal DP, Mishra S, Bishayee A, Sethi G, Kunnumakkara AB (2017) Therapeutic implications of toll-like receptors in peripheral neuropathic pain. Pharmacol Res 115:224–232. https://doi.org/10.1016/j.phrs.2016.11.019
51. Feng T, Yin Q, Weng ZL, Zhang JC, Wang KF, Yuan SY, Cheng W (2014) Rapamycin ameliorates neuropathic pain by activating autophagy and inhibiting interleukin-1beta in the rat spinal cord. J Huazhong Univ Sci Technol Med Sci 34(6):830–837. https://doi.org/10.1007/s11596-014-1361-6
52. Yang YP, Liang ZQ, Gu ZL, Qin ZH (2005) Molecular mechanism and regulation of autophagy. Acta Pharmacol Sin 26(12):1421–1434. https://doi.org/10.1111/j.1745-7254.2005.00235.x
53. Geranton SM, Jimenez-Diaz L, Torsney C, Tochiki KK, Stuart SA, Leith JL, Lumb BM, Hunt SP (2009) A rapamycin-sensitive signaling pathway is essential for the full expression of persistent pain states. J Neurosci 29(47):15017–15027. https://doi.org/10.1523/jneurosci.3451-09.2009
54. Liang L, Tao B, Fan L, Yaster M, Zhang Y, Tao YX (2013) mTOR and its downstream pathway are activated in the dorsal root ganglion and spinal cord after peripheral inflammation, but not after nerve injury. Brain Res 1513:17–25. https://doi.org/10.1016/j.brainres.2013.04.003
55. Richon VM, Sandhoff TW, Rifkind RA, Marks PA (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA 97(18):10014–10019. https://doi.org/10.1073/pnas.180316197
56. Benjamin D, Jost JP (2001) Reversal of methylation-mediated repression with short-chain fatty acids: evidence for an additional mechanism to histone deacetylation. Nucleic Acids Res 29(17):3603–3610
57. Cherng CH, Lee KC, Chien CC, Chou KY, Cheng YC, Hsin ST, Lee SO, Shen CH, Tsai RY, Wong CS (2014) Baicalin ameliorates neuropathic pain by suppressing HDAC1 expression in the spinal cord of spinal nerve ligation rats. J Formos Med Assoc 113(8):513–520. https://doi.org/10.1016/j.jfma.2013.04.007
58. Sanna MD, Guandalini L, Romanelli MN, Galeotti N (2017) The new HDAC1 inhibitor LG325 ameliorates neuropathic pain in a mouse model. Pharmacol Biochem Behav 160:70–75. https://doi.org/10.1016/j.pbb.2017.08.006