Research Paper

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Cognitive impairment in a rat model of neuropathic pain: role of hippocampal microtubule stability

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Abstract

Clinical evidence indicates that cognitive impairment is a common comorbid condition of chronic pain. However, the cellular basis for chronic pain–mediated cognitive impairment remains unclear. We report here that rats exhibited memory deficits after spared nerve injury (SNI). We found that levels of stable microtubule (MT) were increased in the hippocampus of the rats with memory deficits. This increase in stable MT is marked by α-tubulin hyperacetylation. Paclitaxel, a pharmacological MT stabilizer, increased the level of stable MT in the hippocampus and induced learning and memory deficits in normal rats. Furthermore, paclitaxel reduced long-term potentiation in hippocampal slices and increased stable MT (evidenced by α-tubulin hyperacetylation) levels in hippocampal neuronal cells. Intracerebroventricular infusion of nocodazole, an MT destabilizer, ameliorated memory deficits in rats with SNI-induced nociceptive behavior. Expression of HDAC6, an α-tubulin deacetylase, was reduced in the hippocampus in rats with cognitive impairment. These findings indicate that peripheral nerve injury (eg, SNI) affects the MT dynamic equilibrium, which is critical to neuronal structure and synaptic plasticity.

Keywords: Nociceptive behavior, Cognitive impairment, Microtubule, Acetylation, α-tubulin, HDAC6, Hippocampus, LTP, Paclitaxel, Nocodazole

1. Introduction

A growing body of clinical evidence indicates that chronic pain impairs cognitive function.[38,41] An aspect of cognitive impairment evident in patients with chronic pain is deficit in learning and memory.[7,17,27] Despite the clinical evidence, the underlying mechanisms of comorbid cognitive impairment in chronic pain remain to be elucidated.

The hippocampus plays a key role in learning and memory. Accumulating clinical data have shown structural and functional changes in the hippocampus of patients with chronic pain.[18,36,39,40] In rodents, pathological changes in the hippocampus resulting from peripheral nerve injury have been implicated in cognitive deficits.[33,37,40,42]

Microtubules (MTs) are a major cytoskeletal component in neurons.[28] Recent studies reveal a critical role of hippocampal MT dynamics and stability in the learning and memory process.[35,48] Microtubules are essential for the maintenance of neuronal polarity, the formation of the dendritic spines, and receptor trafficking, among other functions.[12,16,28] Microtubule dynamics in mature neurons is fundamental to synaptic plasticity,[28] which are the cellular bases for learning and memory.[50,51] The core structure of MT is composed of α- and β-tubulin heterodimers. α-tubulins of stable and dynamic MTs differ in the type and degree of posttranslational modifications (PTMs).[28,47] α-tubulin is acetylated in stable long-lived MTs.[47] Pharmacological modulation of MT stability with paclitaxel (an MT stabilizer) or nocodazole (an MT destabilizer) affects learning and memory in rodents.[19,48] Furthermore, altered MT dynamic equilibrium in the brain has been implicated in neurological disorders in humans.[21]

The purpose of this study is to investigate the molecular basis for cognitive impairment using a rat model of neuropathic pain (eg, spared nerve injury [SNI]). Acetylation of epsilon-amine groups of lysine residues, a relative common PTM, has a major impact on cellular processes including synaptic plasticity.[10] We analyzed hippocampal tissue using a pan-acetylated-lysine antibody and mass spectrometry. We found that α-tubulin was dominantly hyperacetylated, indicative of increased MT stability, in SNI rats with cognitive impairment. Increasing or restoring MT dynamics in SNI rats by nocodazole, an MT destabilizer, improved cognitive function.

2. Materials and methods

2.1. Experimental animals

Adult male Sprague–Dawley rats (18-28 days old; for patch-clamp recording) weighing 200 to 225 g (~7 weeks) were purchased from Charles River Laboratories. The rats were

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housed in cages with free access to water and food pellets under 12:12 light/dark cycles. Two or 3 rats were housed in one cage. Room temperature was maintained at 19 to 23°C with 40% to 60% humidity. The experimental protocols were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

2.2. Surgical procedures

2.2.1. Spared nerve injury

Rats were anaesthetized with pentobarbital (50 mg/kg, i.p.). Spared nerve injury was produced by ligating and cutting off 2- to 4-mm segments of both common peroneal and tibia nerve branches. Care was taken to spare the sural nerve branch.[15]

2.2.2. Sham operation

Animals in sham groups underwent the same procedure except for nerve ligation and axotomy.

2.2.3. Implantation of a guide cannula for intracerebroventricular microinjection

A guide cannula (Plastics One, Roanoke, VA) was implanted in rats for intracerebroventricular microinjection.[54] The coordinates were 1.5 mm lateral, 1.0 mm posterior from bregma, and 4.0 mm in depth. The cannula location was confirmed histologically at the end of experiment. Paclitaxel and nocodazole (Selleck Chemicals) were dissolved in 5% of 2-hydroxypropyl-β-cyclodextrin (Sigma, St. Louis, MO) for injection. Intracerebroventricular injection (5 μL/injection at 0.5 μL/min) was made using a microsyringe, and the needle was held for 1 minute before retraction.

2.3. Behavioral tests

All behavioral experiments were performed with the investigators being blinded to treatment conditions. Animals were habituated to the test environment for 2 consecutive days (30 minutes per day) before baseline testing. The behavioral tests were conducted between 9 AM and 2 PM

2.3.1. Mechanical allodynia

A von Frey filament was perpendicularly applied to the lateral plantar surface of each hind paw.[15] A threshold force of response (in grams) was defined as the first filament that evoked at least 2 withdrawals of 5 applications.[54]

2.3.2. Acetone test

Fifty microliter of acetone was sprayed directly towards the lateral plantar surface of a hind paw using a microsyringe connected with a blunt needle.[15] The brisk foot withdrawal response after acetone application was considered as a positive response. Both paw withdrawal score[20] and withdrawal duration[2] were measured. The withdrawal was graded based on the following 4-point scale[20]: 0, no response; 1, one time brisk withdrawal or flicking of the paw; 2, repeated (≥2) withdrawal or flicking of the paw; and 3, repeated flicking of the paw plus licking of the paw. The duration (in seconds) of the paw withdrawal was also recorded, and the cutoff was 20 seconds.[2] Each animal underwent 3 trials (once every 5 minutes), and the results were averaged to yield a mean withdrawal score and duration.

2.3.3. Open-field test

The test was performed using a plexiglas square box (57 × 57 cm) with 50-cm-high walls as we previously described.[54] Behaviors were observed for 6 minutes under dim lighting, including a habituation period of 1 minute. The time a rat spent in the central area in open field was recorded for a 5-minute session. Total distance traveled by a rat was measured by SMART video-tracking system (Panlab, Harvard Apparatus, Holliston, MA).[54]

2.3.4. Novel objective recognition test

The test was performed in the same plexiglas box that was used for open-field test. The activity of a rat was recorded by SMART system. Rats were habituated in the box for 5 minutes one day before novel objective recognition (NOR) test. The NOR test consists of a 10-minute training session during which the rat familiarizes itself with 2 identical objects in the box, a 10-minute home cage stay and a 10-minute test session in which a familiar object was replaced with a novel object. The time a rat spent with each object during training and test sessions was recorded using 2 stop watches.[8,29] A recognition index (RI) for each animal was expressed by the ratio TN/(TF + TN)[8,29] (TN: time spent with a novel object, TF: time spent with a familiar object).

2.4. Hippocampal CA3-CA1 long-term potentiation recording, data acquisition, and analysis

2.4.1. Hippocampal slice preparation

Rats (18-28 days old) were anesthetized with pentobarbital (50 mg/kg, i.p.) and decapitated. Hippocampal slices were prepared at room temperature (~25°C) in artificial cerebrospinal fluid (ACSF) as previously described.[54]

2.4.2. Electrophysiological recordings of hippocampal slices

Recordings were made in a fixed-stage, upright microscope equipped with infrared differential interference contrast optics (Olympus, Tokyo, Japan) and an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Field excitatory postsynaptic potentials (fEPSPs) were recorded using ACSF-filled glass pipettes (resistance <2 MΩ) from the outer dendritic regions of CA1. The stimulation was performed with 2 concentric bipolar electrodes (Fred Haer, Brunswick, ME). The test stimuli consisted of biphasic 100-μs pulses of constant current. Baseline responses must be stable for at least 30 minutes before high-frequency stimulation was delivered. Field excitatory postsynaptic potentials were digitized (1 kHz) with the pClamp10 acquisition system (Molecular Devices, LLC, Sunnyvale, CA). The strength and duration of the stimulus pulse were adjusted to elicit a population spike at the cell body layer with an amplitude of 40% to 60% of the maximum spike amplitude. After examining the stability of the responses to a test stimulus that was given every 30 seconds, a tetanus pulse (100 pulses at 100 Hz) was delivered to elicit long-term potentiation (LTP) and responses were recorded for 120 minutes.[6] The fEPSP data were acquired using Clampfit 10 software and analyzed using 1-way analysis of variance (ANOVA). Paclitaxel was dissolved in DMSO and diluted in ACSF.

2.5. Cell culture, western blot, immunostaining, and protein identification by mass spectrometry

HT22 cells were cultured in DMEM media supplemented with 10% FBS and 1% penicillin and streptomycin.[14] Western blots were performed as previously reported[31] using the following primary and secondary antibodies: anti-acetylated-lysine antibody (1:1000; Cell

Signaling, Danvers, MA; #9441), anti-acetyl α-tubulin-Lys40 antibody (1:10,000; 6-11B-1, Santa Cruz), anti-α-tubulin antibody (1:5000; Abcam, Cambridge, MA; ab18251), anti-Tyr-Tub antibody (1:5000; Sigma T9028), anti-HDAC6 antibody (1:1000; Cell Signaling, #7558), anti-ATAT1 antibody (1:1000; Novus Biologicals, Littleton, CO; NBP1-57650), donkey anti-rabbit IgG-HRP (1:20,000; Santa Cruz), and donkey anti-mouse IgG-HRP (1:20,000; Santa Cruz). Immunostaining was performed using FITC-conjugated secondary antibodies (Jackson Immuno Research Labs) as we described previously.[54] Protein identification was conducted by Science Core Facility at Harvard University (http://proteomics.fas.harvard.edu/). Briefly, after in-gel trypsin digestion, a sample was submitted for single LC-MS/MS experiment that was performed on a LTQ Orbitrap Elite (Thermo Fischer, Waltham, MA) equipped with Waters NanoAcquity HPLC pump (Milford, MA). Raw data were submitted for analysis in Proteome Discoverer 2.1.0.81 (Thermo Fischer) software. Assignment of MS/MS spectra was performed using the Sequest HT algorithm by searching the data against UniprotKB/Swissprot database.

2.6. Statistical analysis of data

Behavioral data were analyzed using 2-way ANOVA repeated across time points or 1-way ANOVA as appropriate.[31] Post hoc Waller–Duncan K-ratio *t* test was performed to determine the source(s) of differences. One-way ANOVA was used to analyze the data from western blots. Graphpad5 software was used for the statistical analyses. All data were expressed as mean ± SEM, and the statistically significant level was set at $P < 0.05$.

3. Results

3.1. Spared nerve injury–induced cognitive deficits

Unilateral SNI in rats produced prolonged mechanical allodynia and cold hyperalgesia in the ipsilateral hind paw as compared to sham controls (**Figs. 1A–C**). In the open-field test, the distance traveled by SNI rats was similar to that of sham rats (**Figs. 1D and E**) suggesting that the surgery by itself did not affect the locomotor activity. It was also noticeable that SNI rats spent less time in the central area in the open field than sham rats at 14 days after surgery (**Figs. 1D and F**). To evaluate the effect of nociception on cognitive function, we subjected both SNI and sham rats to NOR test. At 4 days after surgery, SNI and sham rats had a similar RI for novel object in the testing phase (**Fig. 2A**). However, SNI rats had a lower RI for novel object than sham rats when tested again at 14 days after surgery (**Fig. 2B**). In NOR test, a normal rat would remember the objects being exposed during the training phase. In the testing phase, the rat would spend more time exploring a novel object than a familiar object, which is expressed as a higher RI index for novel object.[29] These data suggest that as SNI-induced nociception transitioned into a chronic phase, animals exhibited cognitive impairment.

3.2. α-tubulin dominated the increase in protein acetylation in the hippocampus of rats with prolonged nociceptive behavior

Acetylation of proteins other than histones affects synaptic plasticity.[10] Both structural and functional abnormalities in the hippocampus have been observed in patients with chronic pain and in rodents with prolonged nociceptive behavior.[40] We examined the spared nerve injury–mediated changes in protein acetylation in the hippocampus using a pan-acetyl-lysine antibody in western blot. We found that there was a dominant band sized between 50 and 65 kDa with significantly increased acetylation for hippocampal tissues isolated from SNI rats at 14 days after surgery (**Fig. 3A**). To identify the protein(s), we purified the protein(s) from the hippocampal lysate by size fractionation and immunoprecipitation with anti-acetylated lysine antibody and analyzed the protein(s) with mass spectrometry. The dominant

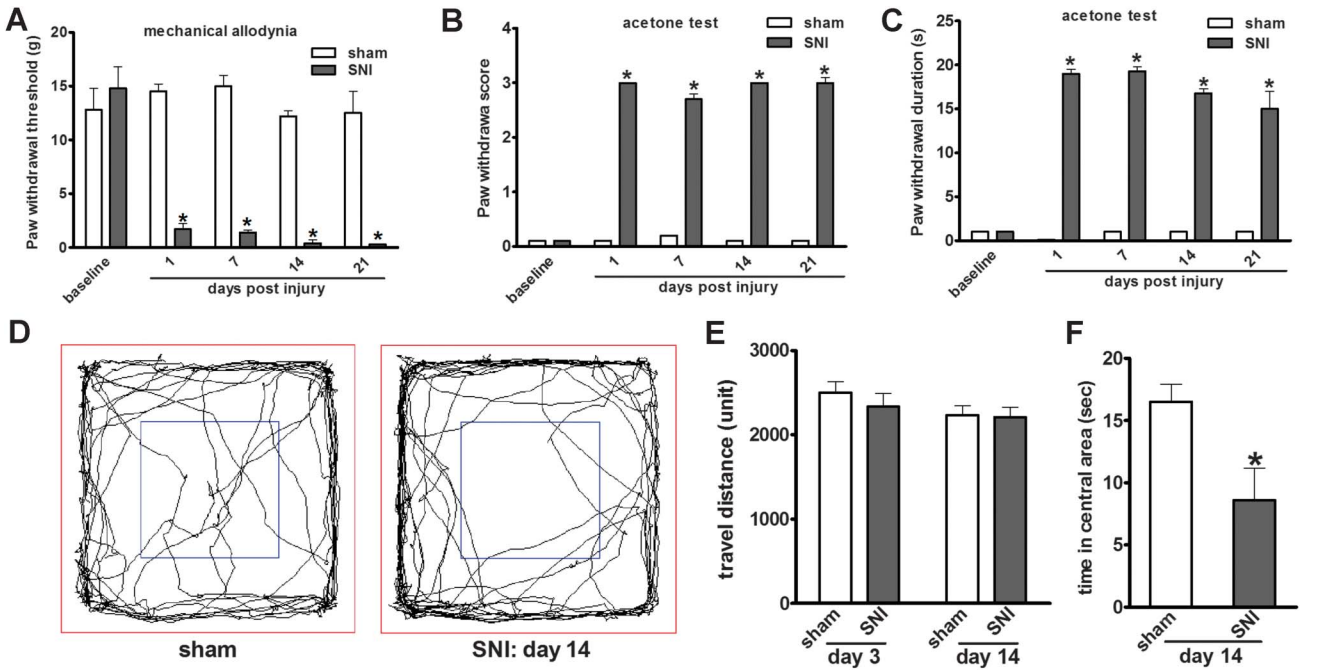

Figure 1. Spared nerve injury rats showed prolonged nociceptive behavior while maintained normal locomotor activity. (A–C) Spared nerve injury–induced mechanical allodynia was demonstrated as reduced mechanical withdrawal threshold (A), and cold hyperalgesia was manifested as increased withdrawal score (B) and duration in acetone test (C). (n = 8/group, *$P < 0.05$, sham vs SNI). (D) A typical locomotion pattern, recorded by a video tracing program, in an open-field test from a sham-operated or SNI rat at 3 and 14 days after surgery. (E) At 14 days after surgery, the total travel distances were similar between SNI rats and sham rats in open field. (F) Spared nerve injury rats displayed thigmotaxis, and they spent less time in the central area than sham rats (n = 8/group, *$P < 0.05$, sham vs SNI). SNI, spared nerve injury.

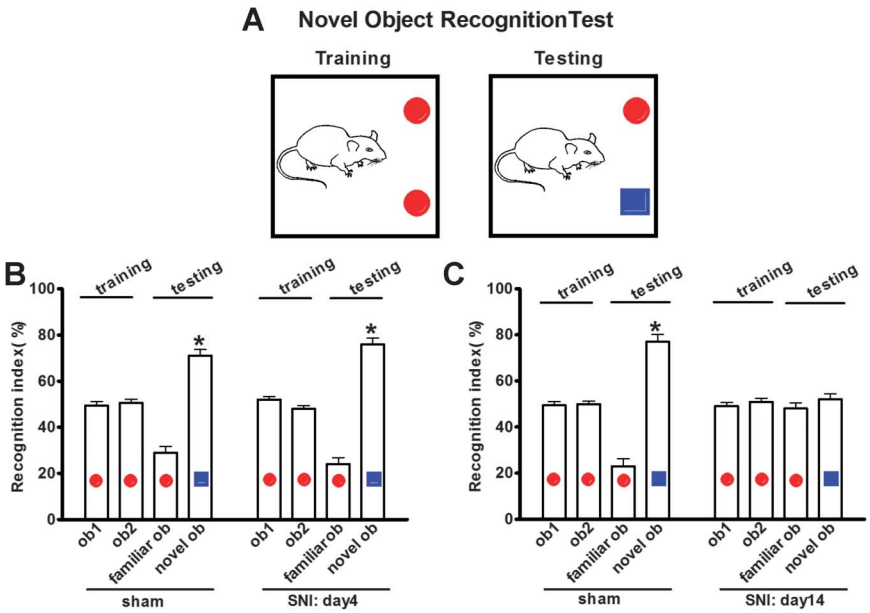

Figure 2. Chronic nociception impaired cognitive function in SNI rats in NOR test. (A) A schematic drawing illustrates the training and testing phases of NOR test. (B) At 4 days after surgery, both sham and SNI rats spent equal amount of time exploring 2 identical objects (ob1 and ob2) during training phase. The 2 groups of rats had a similar recognition index (RI) for both objects. In the testing phase, both sham and SNI rats spent more time exploring the novel object (novel ob) than the familiar object (ob1 or ob2). Thus, rats from both groups had a higher RI for novel object than familiar object. (C) At 16 days after surgery, sham and SNI rats had the same performance in a training phase. In the testing phase, sham group had a higher RI for novel object than for familiar object. However, SNI rats had similar RI for both novel and familiar objects. Data values are expressed as mean ± SEM (n = 8/group, $^*P < 0.01$, familiar ob vs novel ob). NOR, novel objective recognition; SNI, spared nerve injury.

protein from hippocampal lysate was identified as α-tubulin (Fig. 3B, Supplement Table 1; available online at http://links.lww.com/PAIN/A565). Lys40 is the canonical site for α-tubulin acetylation.[28] We used acetylated α-tubulin (Lys40 or K40) antibody to confirm the identity of this protein (**Fig. 3C**).

3.3. Spared nerve injury increased stable microtubule in the hippocampus

Acetylated α-tubulin exists in stable long-lived MTs.[25,47] Analysis of hippocampal tissue showed an increased level of acetylated α-tubulin at 14 days after SNI injury (**Fig. 4A**). This increase was not caused by an increased level of tubulin protein expression (**Fig. 4A**). Next, we analyzed the level of tyrosinated α-tubulin and demonstrated that spared nerve injury increased the amount of stable MT as tyrosination of tubulin (Tyr-T) is restricted to soluble α-tubulin dimmers.[47] In the hippocampus, tyrosinated α-tubulin was decreased in SNI rats at 14 days after injury compared with sham and other time points of SNI (**Fig. 4**). These data suggest that the amount of stable MT was increased in the hippocampus of rats with prolonged nociceptive behavior.

3.4. Increased stable microtubule in the hippocampus-impaired cognitive function

We examined cognitive function in rats with pharmacologically increased stable MTs in the hippocampus. We used paclitaxel, an MT stabilizer, which stabilizes MTs by enhancing polymerization.[48] When tested 12 hours after brain microinjection, the paclitaxel-treated rats had a lower RI for a novel object than those with vehicle treatment, indicating cognitive impairment (**Fig. 5A**). To search for a synaptic mechanism for the effect of an increased stable MT level on cognitive impairment, we assessed the effect of increased stable MTs on LTP in hippocampal slices. Test stimulation intensity had an average voltage of 2.3 ± 0.87 mV, and the fEPSP had a slope of −3.17 ± 0.56 mV/seconds (42.4.6% ± 0.68% of the maximal fEPSP slope) for control group and −2.23 ± 0.68 mV/seconds for paclitaxel group, respectively. Paclitaxel treatment reduced LTP at CA3-CA1 synapses in the hippocampus (**Figs. 5B–D**). Thus, the stabilization of MT by paclitaxel inhibited hippocampal LTP. In addition, paclitaxel treatment increased stable MTs in hippocampal HT22 cells, as paclitaxel dose dependently increased acetyl-α-tubulin level (**Figs. 6A and B**). These results confirm that an increase in stable MTs in the hippocampus is linked to impaired learning and memory.

3.5. Decreasing hippocampal stable microtubules by nocodazole in spared nerve injury rats ameliorated cognitive impairment

To examine whether decreasing stable MTs in the hippocampus of SNI rats would improve cognitive function, we used brain microinjection of nocodazole, an antineoplastic agent that induces MT's depolymerization and decreases stable MT levels.[48] We treated the SNI rats with nocodazole (intracerebroventricular [i.c.v.] 5 μL of 30 nM), and NOR test was performed at 12 hours after brain microinjection. Compared with vehicle treatment, nocodazole treatment increased RI for novel object in SNI rats (**Fig. 7A**). Nocodazole treatment did not reduce mechanical allodynia in SNI rats (**Fig. 7B**). Western blot analysis of hippocampal tissue showed that nocodazole treatment reduced acetyl-α-tubulin levels in the hippocampus of SNI rats (**Fig. 7C**). Nocodazole treatment of HT22 cells decreased MT stability as indicated by decreased acetyl-α-tubulin levels after the treatment (**Figs. 8A and B**). To demonstrate that the balance between MT dynamics and stability is crucial for cognitive function and hyperdynamic MT impairs cognitive function, we showed that

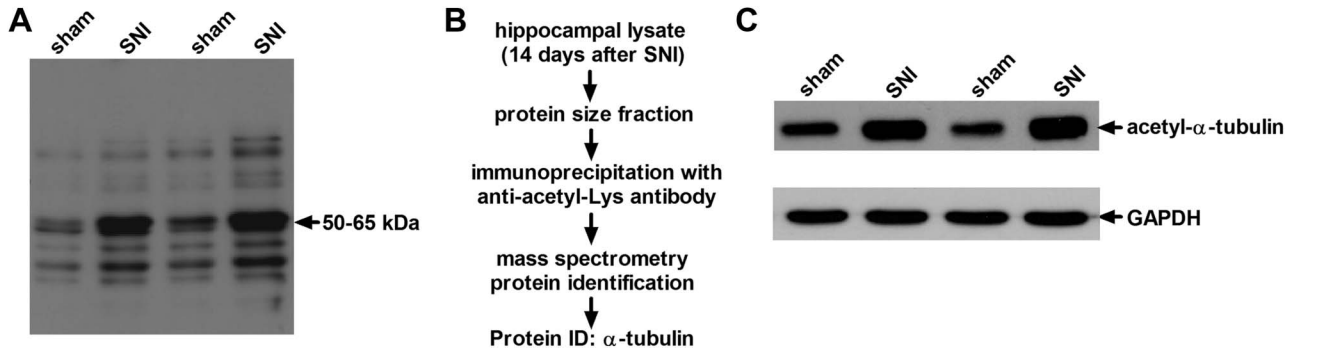

Figure 3. α-tubulin acetylation was increased in the hippocampus of SNI rats. (A) Western blot analysis of hippocampal tissue lysates from sham and SNI (day 14) rats using pan-acetyl-lysine antibody. A dominant band sized between 50 and 65 kDa with significantly increased acetylation was observed in SNI (day 14) rats. (B) Flow chart illustrates protein identification process. (C) Western blot analysis of hippocampal tissue lysates from sham and SNI (day 14) rats using anti-acetyl-α-tubulin-ly40 antibody. The amount of acetyl-α-tubulin (α-tubulin K40) (50 kDa) was increased in the hippocampus of SNI rats. SNI, spared nerve injury.

nocodazole-treated naive rats had a lower RI value for novel object in NOR test (**Fig. 8C**). These results are consistent with previous studies showing that nocodazole impairs cognitive function[19] and abrogates LTP.[3] Mechanical sensitivity was not affected by nocodazole treatment in rats (**Fig. 8D**).

3.6. HDAC6 expression was decreased in the hippocampus of spared nerve injury rats

The major enzymes involved in α-tubulin acetylation and deacetylation are α-tubulin acetyltransferase ATAT1 and HDAC6, respectively.[24] We examined both ATAT1 and HDAC6 protein expression in the hippocampus of sham and SNI rats. Western blot analysis did not reveal differences in ATAT1 expression in the hippocampus between sham and SNI rats (**Fig. 9B**). However, HDAC6 expression was decreased in the hippocampus of SNI rats compared with sham rats as demonstrated by both western blot and immunohistology (**Figs. 9A and B**). In the hippocampus of SNI rats, decreased HDAC6 expression correlated with increased α-tubulin acetylation (**Fig. 4**).

4. Discussion

Patients with chronic pain suffer from cognitive impairment, which poses a major obstacle to daily activities and rehabilitation.[38,41] Clinical studies have revealed anatomical and functional changes in brain regions involved in cognitive and pain processing among patients with chronic pain.[17,18,36,39] However, cellular mechanisms underlying chronic pain–induced cognitive impairment remain elusive. Peripheral nerve injury induces learning and memory deficits after peripheral nerve injury in rodents,[33,40,42] which makes it possible to explore the molecular basis for chronic pain–induced cognitive impairment.

In this study, we showed that SNI rats with prolonged nociceptive behavior exhibited recognition memory deficits when subjected to NOR test, which is consistent with a recent study showing that rats with nociceptive behavior resulting from spinal nerve ligation failed to recognize novel object in NOR test.[37] Novel objective recognition is a task that involves locomotor and exploratory activity.[29] Anxiety and depression, which are known to be comorbid with nociceptive behavior in rodents,[54] and nerve injury may affect an animal's activity in NOR test. Analysis of baseline total locomotor activity suggests that SNI injury did not affect locomotor activity. Spared nerve injury rats showed thigmotaxis in the open field, which is consistent with previous studies.[43,54] To minimize the impact of thigmotactic behavior of SNI rats on NOR test, the objects were placed close to the edge of the open field. Thus, the difference in RI for novel object between sham and SNI rats was unlikely due to this potential confounding factor. Novel objective recognition test has been widely used to assess learning and recognition memory, in which the hippocampus plays an essential role.[8,11] In short, spared nerve injury impaired the hippocampal-dependent cognitive function.

Similar to phosphorylation of serine, threonine, or tyrosine, acetylation of lysine is an important reversible protein PTM. The

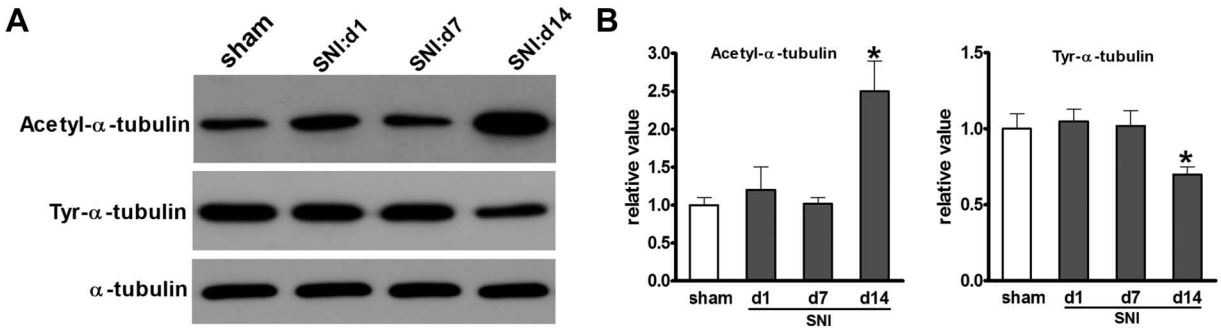

Figure 4. Hippocampal α-tubulin posttranslational modifications were changed after SNI. (A) Western blot analysis of hippocampal tissue lysates from sham and SNI (day 1, day 7, and day 14) rats using anti-acetyl-α-tubulin (α-tubulin K40) antibody and anti-tyrosinated α-tubulin antibody (acetyl-, Tyr-α-tubulin: 50 kDa). (B) Levels of acetyl-α-tubulin were increased at 14 days after SNI. The levels of tyrosinated α-tubulin were decreased at 14 days after SNI (n = 4/group, $^*P < 0.05$. sham vs SNI day 14). SNI, spared nerve injury.

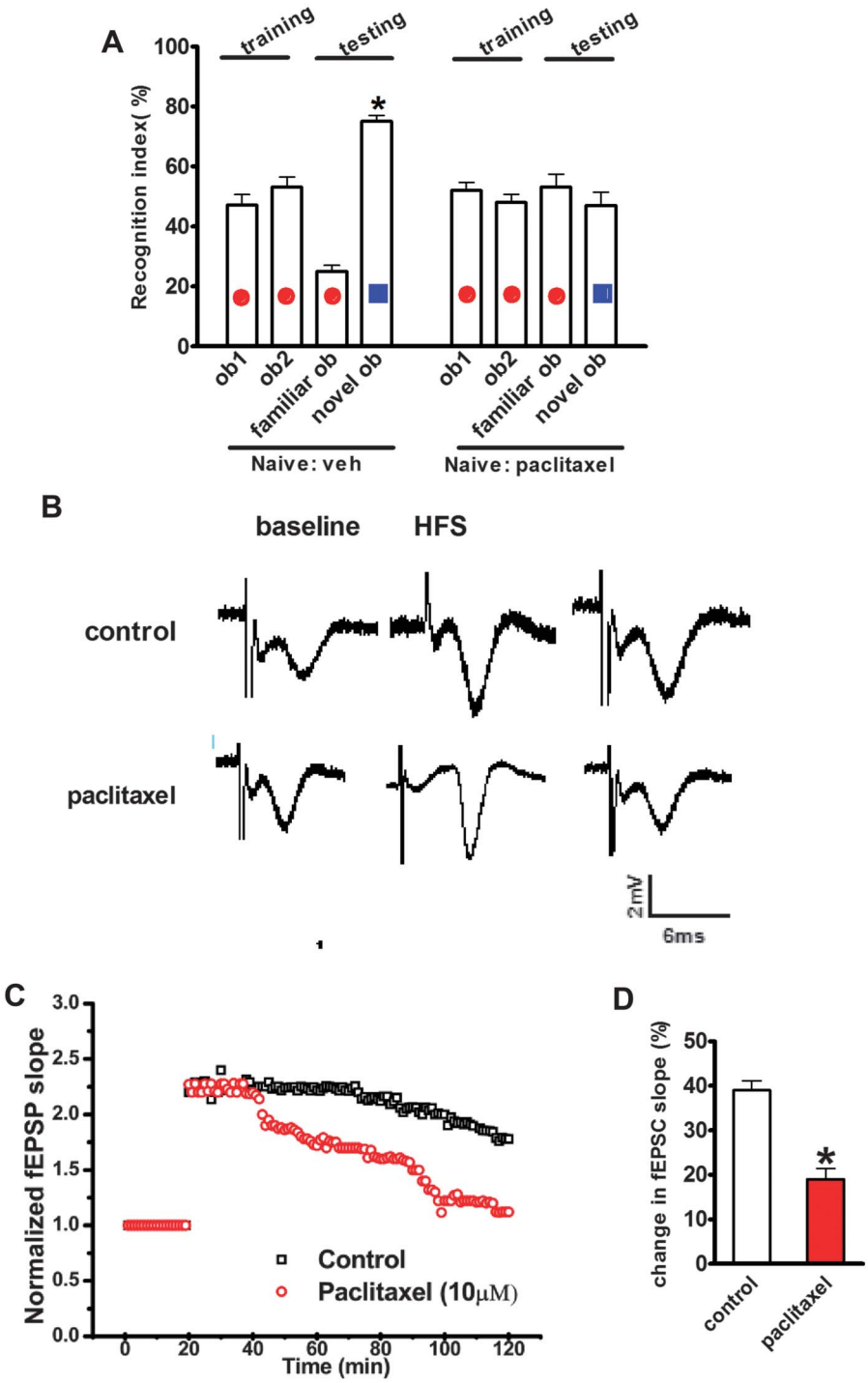

Figure 5. Increased MT stability impaired learning and memory. (A) Brain microinjection of the MT stabilizer paclitaxel did not affect the recognition index (RI) of naive rats during the training phase in NOR test. During the testing phase, vehicle (veh)-treated naive rats had a higher RI for the novel object than the familiar object, whereas paclitaxel-treated rats failed to recognize the novel object. Data values are expressed as mean ± SEM. (n = 8/group, $^*P < 0.01$, familiar ob vs novel ob). (B) Representative traces of fEPSP for prestimulation baseline, high-frequency stimulation (HFS), and application of paclitaxel (10 μM). (C) Averaged field EPSP data. High-frequency stimulation was given at 21 minutes, paclitaxel was applied at 41 minutes, and signal was followed for up to 120 minutes. The application of paclitaxel reduced LTP at CA3-CA1 field of the hippocampus. (D) Summary of changes in fEPSPs (from 20 to 120 minutes) shown in (C). Data values are expressed as mean ± SEM (n = 6, $^*P < 0.01$, control vs paclitaxel). fEPSP, field excitatory postsynaptic potential; NOR, novel objective recognition; MT, microtubule.

brain is rich in acetylated proteins, which are primarily involved in neuronal signal transmission.[34] We found that α-tubulin was hyperacetylated in SNI rats with cognitive impairment. Increased MT stability is marked by increased acetyl-α-tubulin. Microtubules, a main cytoskeleton component, are markedly enriched in brain neurons[12] and play an essential role in neuronal functions.[26,52] Dendritic spines are the postsynaptic component of excitatory neurons. Microtubule dynamics plays a critical role in dendritic spine remodeling and MT-mediated transporting of cargoes into and out of dendritic spines.[16,22,23,26,52] In SNI rats

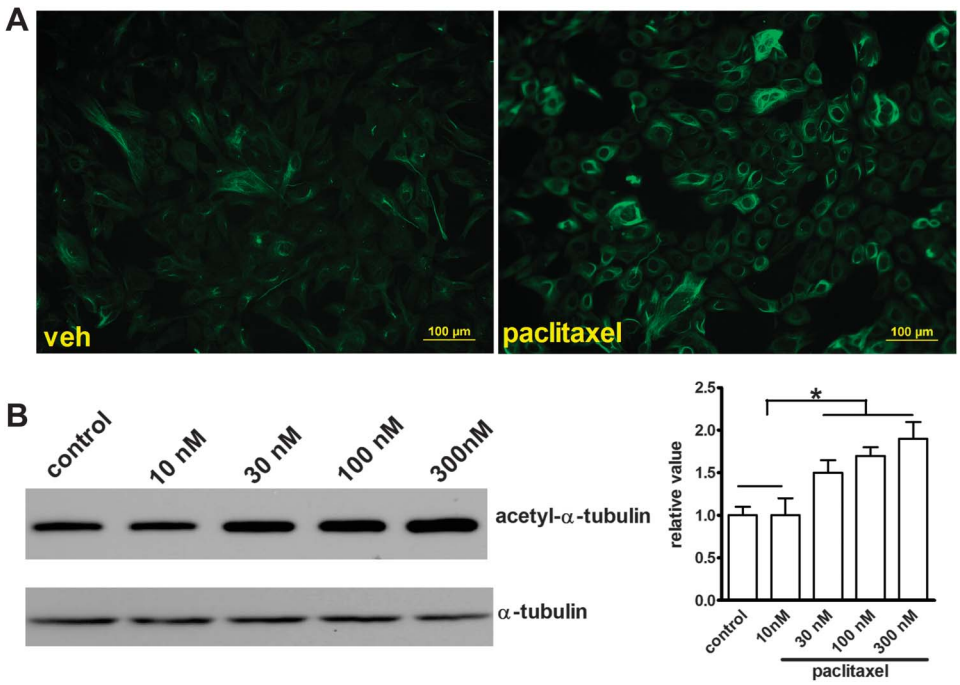

Figure 6. Paclitaxel increased α-tubulin acetylation in hippocampal HT22 cells. (A) Immunostaining of acetyl-α-tubulin of HT22 cells treated with vehicle (veh) or paclitaxel (300 nM) for 12 hours. (B) Western blot analysis of acetyl-α-tubulin (50 kDa) of HT22 cells treated with vehicle control or paclitaxel (10, 30, 100 and 300 nM). Triplicates, $^*P < 0.05$.

with spatial memory deficits, the dendrite lengths and spine densities are reduced significantly in hippocampal neurons.[33] Our finding that SNI caused an increase in hippocampal MT stability suggests a molecular basis of chronic pain–induced cognitive impairment involving dendritic spine remodeling in the hippocampus.

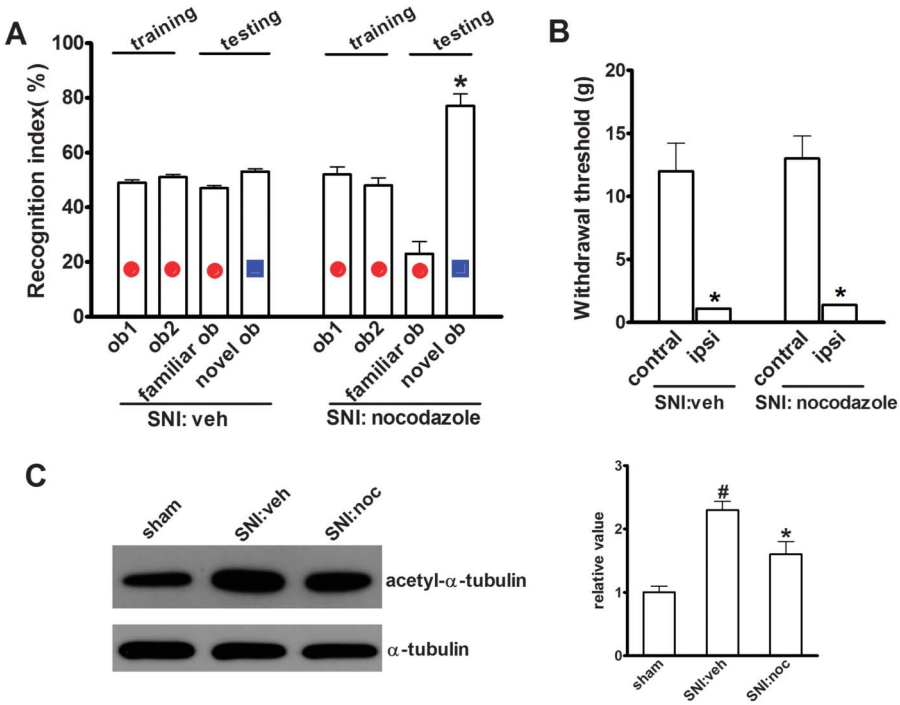

Figure 7. Nocodazole improved cognitive function in SNI rats. (A) Brain microinjection of nocodazole increased the recognition index (RI) for novel object in SNI rats (14 days after injury) in NOR test. (n = 8/group, $^*P < 0.05$, familiar ob vs novel ob). (B) Nocodazole treatment did not improve mechanical allodynia in SNI rats. (n = 8/group, $^*P < 0.05$, contralateral vs ipsilateral). (C) Western blot analysis of hippocampal tissue showing that nocodazole treatment decreased acetyl-α-tubulin levels in SNI (d14) rats. (n = 4/group, $^*P < 0.05$, SNI:veh vs SNI:nocodazole; $\#P < 0.05$, sham vs SNI:veh). NOR, novel objective recognition; SNI, spared nerve injury.

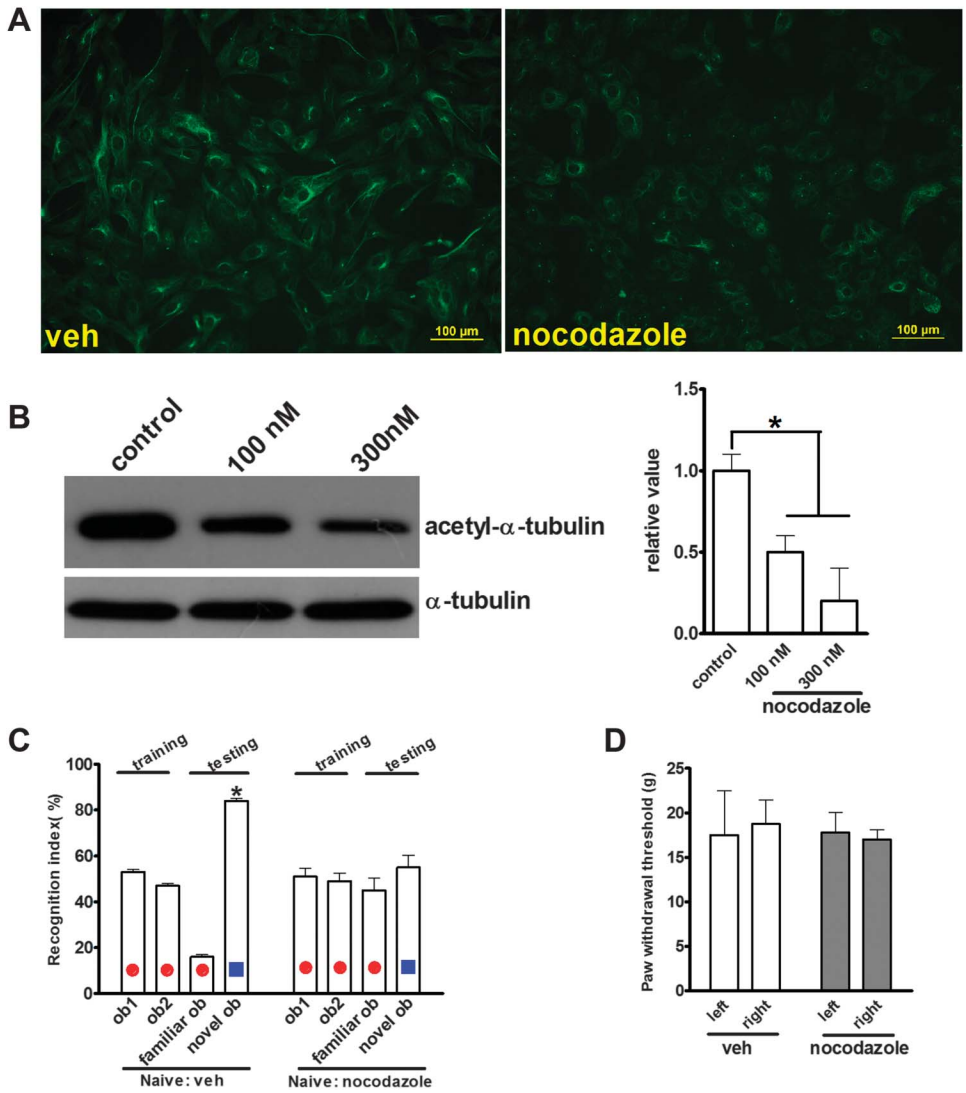

Figure 8. Nocodazole increased MT dynamicity and affected cognitive function. (A) Immunostaining of acetyl-α-tubulin of HT22 cells treated with vehicle (veh) or nocodazole (300 nM) for 16 hours. (B) Western blot analysis of acetyl-α-tubulin (50 kDa) of HT22 cells treated with vehicle control or nocodazole (100 and 300 nM). Triplicates, $^*P < 0.05$. (C) Naive rats injected intracerebroventricular with nocodazole failed to recognize a novel object in NOR test (n = 4-5/group, $^*P < 0.01$, familiar ob vs novel ob). (D) Mechanical sensitivity was similar between naive rats treated with vehicle or nocodazole. NOR, novel objective recognition; MT, microtubule.

Both experimental and molecular modeling studies have revealed critical roles for MT in synaptic plasticity and memory.[12,13,16,26,28] Pharmacologically or genetically mediated changes in MT dynamics/stability affect contextual learning and associative memory consolidation.[19,35,46] Consistent with these studies, we found that the MT-stabilizer paclitaxel impaired learning and memory of a normal rat in NOR test when administered into the hippocampus. Furthermore, paclitaxel increased α-tubulin acetylation in hippocampal HT22 cells and inhibited LTP in hippocampal slices. Long-term potentiation at hippocampal synapses has been considered as the cellular basis for learning and memory.[6] A recent study demonstrated reduced hippocampal LTP in rodents with peripheral nerve injury–mediated memory deficits.[42] It is possible that increased MT stability in the hippocampus, leading to inhibition of LTP in SNI rats, contributed to the cognitive impairment. Conversely, treatment with nocodazole, an MT destabilizer, improved cognitive function of SNI rats in NOR test, lending further support to this notion. It is of note that decreased MT stability relative to normal state causes cognitive deficits as well.[4,19] Fanara et al. (2010) found that nocodazole (i.c.v.) caused cognitive deficits in mice and used ^2H labeling to quantify the increase in free tubulin dimers in the hippocampus of these mice. Similarly, our data show that nocodazole decreased acetyl-α-tubulin levels in HT22 cells and caused cognitive impairment in naive rats. In short, perturbation of the normal (optimal) MTs stability or dynamicity in the hippocampus impairs cognitive function. In other words, cognitive function requires an optimal balance between MT stability and dynamicity.[4,19,48] We did not observe a concurrent amelioration of nociceptive behavior in SNI rats after nocodazole treatment. The hippocampus participates in affective pain processing.[32] However, a direct role for the hippocampus in sensory function remains to be established.[32] Peripheral nerve injury may modulate molecular signals regulating dynamics of the MT network, which in turn contributes to alterations in neuronal plasticity in rodents with prolonged nociceptive behavior.

In this study, we administered drugs through (i.c.v.) injection, which could affect brain regions other than the hippocampus. We

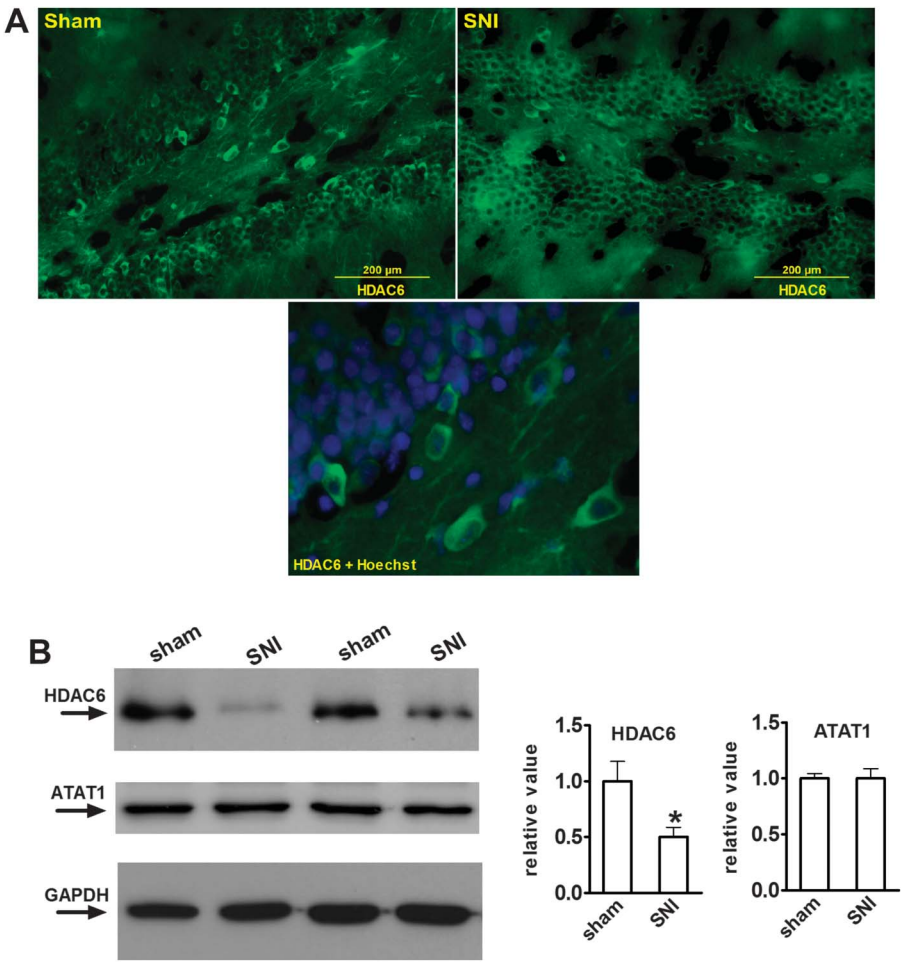

Figure 9. HDAC6 expression was reduced in the hippocampus of SNI rats. (A) Representative images of HDAC6 immunostaining of the hippocampus sections from sham or SNI rats harvested at 14 days after surgery. Hoechst (blue) and anti-HDAC6 antibody (green) costaining showed cytoplasm expression of HDAC6. (B) Western blot analysis of HDAC6 protein (160 kDa) and ATAT1 protein (α-tubulin acetyltransferase 1: 36 kDa) in hippocampal tissues of sham and SNI rats at 14 days after surgery. (n = 5/group, $^*P < 0.05$). SNI, spared nerve injury.

consider that the behavioral outcome of nocodazole (i.c.v.) treatment was mediated by changes in the hippocampal MT stability as we did not observe significant changes in acetyl-α-tubulin levels in other brain regions (unpublished data). Preferably, hippocampal infusion of drugs can be used to minimize the effects of drug on other brain regions and to identify the role of a specific hippocampal subfield in SNI-induced cognitive impairment.

The intrinsic dynamic instability of MTs is essential for neuronal plasticity.[12,16] Microtubule dynamics/stability and function are modulated by MT-associated proteins (eg, MAP2, Tau),[5] MT regulatory proteins,[47,48] and reversible PTMs of tubulin subunits.[25] HDAC6 is an MT-associated deacetylase, which is responsible for acetylation of α-tubulin.[24] HDAC6 is localized exclusively in the cytoplasm and is highly expressed in the hippocampus.[24,45] Our immunohistological data showed the cytoplasm expression of HDAC6 in the hippocampus. Furthermore, our data indicate that decreased hippocampal HDAC6 expression levels correlated with α-tubulin hyperacetylation in SNI rats with cognitive deficits. Taken together, decreased hippocampal HDAC6 expression may be one of the underlying molecular mechanisms for increased MT stability after peripheral never injury.

Because the ratio of stable to instable MT is important for normal neuronal function, disruption of MT dynamics has been implicated in neurobiological diseases. For example, reduced MT stability is linked to neurodegenerative diseases such as Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, and progressive supranuclear palsy.[9,21,30,53] Changes in MT dynamics in the hippocampus are also linked to schizophrenia, stress, depression, and alcohol addiction.[1,44,49] Improper regulation of MT stability has been associated with age-related memory loss in rodents.[48] Although studying MT dynamics directly in living animals is exceedingly difficult,[16] our work indicates that chronic pain, such as other neurobiological diseases, affects this basic cellular structure critical to neuronal synaptic plasticity.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Author contributions: Z. You and J. Mao conceived the project, designed the experiments, and wrote the manuscript. S. Zhang

performed the LTP experiment. Z. You, S. Shen, W. Ding, L. Yang, G. Lim, J.T. Doheny, and S. Tate conducted the experiments. J. Yang performed protein, statistical, and image analysis and manuscript preparation. L. Chen helped with manuscript preparation.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A565.

Supplemental video content

Video content associated with this article can be found online at http://links.lww.com/PAIN/A564.

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