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Cognitive Impairment in a rat model of neuropathic pain: Role of Hippocampal Microtubule Stability

Zerong You^{a,1}, Shuzhuo Zhang^{b,1}, Shiqian Shen^{a,1}, Jinsheng Yang^a, Weihua Ding^{a,c}, Liuyue Yang^a, Grewo Lim^a, Jason T Doheny^a, Samuel Tate^a, Lucy Chen^a, and Jianren Mao^{a,*}

^aMGH Center for Translational Pain Research, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

^bBeijing Institute of Pharmacology and Toxicology, 27 Tai-Ping Road, Beijing, China, 100850

^cThe First Affiliated Hospital of Zhejiang University, 79 Qingchun Road, Hangzhou, China, 310003

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 (LTP) in hippocampal slices and increased stable MT (evidenced by α -tubulin hyperacetylation) levels in hippocampal neuronal cells. Intracerebroventricular infusion of nocodazole, a 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 (e.g. 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

*Correspondence should be addressed to Dr. Jianren Mao, MGH Center for Translational Pain Research, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114. jmao@mgh.harvard.edu.

¹These authors contributed equally to this work

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Author contributions:

Z.Y. and J.M. conceived the project, designed the experiments and wrote the manuscript. S. Z. performed the LTP experiment. Z.Y., S.S., W.D., L.Y., G.L., J.D. and S.T. conducted the experiments. J.Y. performed protein, statistical and image analysis and manuscript preparation. L.C. helped with manuscript preparation.

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 deficits 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]. MTs are essential for the maintenance of neuronal polarity, the formation of the dendritic spines, and receptor trafficking, among other functions [12; 16; 28]. MT dynamics in mature neurons is fundamental to synaptic plasticity [28], which is 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 post-translational 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]. Further, 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 (e.g. SNI). Acetylation of epsilon-amine groups of lysine residues, a relative common post-translational modification (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, a 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 200–225 g (~7 weeks) were purchased from Charles River Laboratories. The rats were housed in cages with free access to water and food pellets under 12:12 light/dark cycles. Two or three rats were housed in one cage. Room temperature was maintained at 19–23°C with 40–60% 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 (SNI) was produced by ligating and cutting off 2–4 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) for injection. Intracerebroventricular injection (5 μ L/injection at 0.5 μ L/min) was made using a microsyringe and the needle was held for 1 min before retraction.

2.3. Behavioral tests

All behavioral experiments were carried out with the investigators being blinded to treatment conditions. Animals were habituated to the test environment for two 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 two withdrawals out of five applications [54].

2.3.2.—Acetone test: fifty μ L 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; 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 three trials (once every 5 min) and the results were averaged to yield a mean withdrawal score and duration.

2.3.3. Open field test (OFT)—The test was carried out 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) [54].

2.3.4. Novel objective recognition (NOR) test—The test was carried out in the same Plexiglas box which was used for OFT. The activity of a rat was recorded by SMART system. Rats were habituated in the box for 5 min one day before NOR test. The NOR test consists of a 10-min training session during which the rat familiarizes itself with two identical objects in the box, a 10-min home cage stay and a 10-min 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 two 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 LTP recording, data acquisition and analysis

2.4.1. Hippocampal slice preparation—Rats (18 to 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 (IR-DIC) optics (Olympus, Tokyo, Japan) and an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Field excitatory postsynaptic potentials (fEPSPs) were recorded using artificial cerebrospinal fluid-filled glass pipettes (resistance < 2 MΩ) from the outer dendritic regions of CA1. The stimulation was performed with two 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 min before high frequency stimulation (HFS) was delivered. fEPSPs were digitized (1 kHz) with the pClamp10 acquisition system (Molecular Devices, LLC, Sunnyvale, CA, USA). The strength and duration of the stimulus pulse were adjusted to elicit a population spike at the cell body layer with an amplitude of 40–60% of the maximum spike amplitude. After examining the stability of the responses to a test stimulus that was given every 30 s, a tetanus pulse (100 pulses at 100 Hz) was delivered to elicit LTP and responses were recorded for 120 min [6]. The fEPSP data were acquired using Clampfit 10 software and analyzed using one-way 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 carried out as previously reported [31] using the following primary and secondary antibodies: anti-acetylated-lysine antibody (1:1000; Cell Signaling, #9441), anti-acetyl α-tubulin-Lys40 antibody (1:10,000; 6-11B-1, Santa Cruz), anti-α-tubulin antibody (1:5000; Abcam, ab18251), anti-Tyr-Tub antibody (1:5000; Sigma T9028), anti-HDAC6 antibody (1:1000; Cell Signaling, #7558), anti-ATAT1 antibody (1:1000; Novus Biologicals, 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) 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 two-way analysis of variance (ANOVA) repeated across time points or one-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 $\pm$ SEM and the statistically significant level was set at $P < 0.05$.

3. Results

3.1. Spared nerve injury induced cognitive deficits

Unilateral spared nerve injury (SNI) in rats produced prolonged mechanical allodynia and cold hyperalgesia in the ipsilateral hind paw as compared to sham controls (Fig 1A, B, and C). In the open field test, the distance traveled by SNI rats was similar to that of sham rats (Fig 1D, 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 (Fig 1D, F). To evaluate the effect of nociception on cognitive function, we subjected both SNI and sham rats to novel object recognition (NOR) test. At 4 days after surgery, SNI and sham rats had a similar recognition index (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 chronic pain patients 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 protein from hippocampal lysate was identified as α -tubulin (Fig 3B, Supplement Table 1). 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 (MT) in the hippocampus

Acetylated α -tubulin exists in stable long-lived microtubules [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 dimers [47]. In the hippocampus, tyrosinated α -tubulin was decreased in SNI rats at 14 days after injury compared to 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 MT in the hippocampus impaired cognitive function

We examined cognitive function in rats with pharmacologically increased stable MTs in the hippocampus. We used paclitaxel, a microtubule stabilizer, which stabilizes microtubules by enhancing polymerization [48]. When tested 12 h after brain microinjection, the paclitaxel-treated rats had a lower recognition index (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 long-term potentiation (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/s ($42.4.6\% \pm 0.68\%$ of the maximal fEPSP slope) for control group and -2.23 ± 0.68 mV/s for paclitaxel group respectively. Paclitaxel treatment reduced LTP at CA3-CA1 synapses in the hippocampus (Figure 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 (Fig 6A, 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 MTs by nocodazole in SNI 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 anti-neoplastic agent that induces microtubule's depolymerization and decreases stable MT levels [48]. We treated the SNI rats with nocodazole (i.c.v. 5 μ L of 30 nM) and NOR test was performed at 12 h after brain microinjection. Compared with vehicle treatment, nocodazole treatment increased recognition index (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 (Fig 8A, 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 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 SNI 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 (Fig 9A, B). In the hippocampus of SNI rats, decreased HDAC6 expression correlated with increased α -tubulin acetylation (Fig 4).

4. Discussion

Chronic pain patients 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 chronic pain patients [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 novel object recognition (NOR) test, which is consistent with a recent study showing that rats with nociceptive behavior resulting from spinal nerve ligation (SNL) failed to recognize novel object in NOR test [37]. NOR 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. SNI 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. NOR 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 posttranslational modification (PTM). The 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 microtubule (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. MT 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 with spatial memory deficits, the dendrite lengths and spine densities are reduced significantly in hippocampal neurons [33]. Our finding that spared nerve injury (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.

Both experimental and molecular modeling studies have revealed critical roles for microtubule 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. LTP 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, a microtubule 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 P *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) microtubules (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 microtubule network, which in turn contributes to alterations in neuronal plasticity in rodents with prolonged nociceptive behavior.

In this study, we administered drugs through intracerebroventricular (i.c.v.) injection, which could affect brain regions other than the hippocampus. We 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 microtubules is essential for neuronal plasticity [12; 16]. Microtubule (MT) dynamics/stability and function are modulated by MT-associated proteins (e.g. MAP2, Tau) [5], microtubule regulatory proteins [47; 48], and reversible post-translational modifications (PTMs) of tubulin subunits [25]. HDAC6 is a microtubule-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 microtubule stability after peripheral nerve injury.

Because the ratio of stable to unstable 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's disease, Parkinson's 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 microtubule stability has been associated with age-related memory loss in rodents [48]. Even though studying MT dynamics directly in living animals is exceedingly difficult [16], our work indicates that chronic pain, like other neurobiological diseases, affects this basic cellular structure critical to neuronal synaptic plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. SNI rats showed prolonged nociceptive behavior while maintained normal locomotor activity

(A–C) SNI-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). ($n = 8/\text{group}$, $*p < 0.05$, 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) SNI rats displayed thigmotaxis, and they spent less time in the central area than sham rats. ($n = 8/\text{group}$, $*p < 0.05$, sham vs. SNI)

Fig. 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 two identical objects (ob1 and ob2) during training phase. The two 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 $\pm$ SEM. (n=8/group, * p <0.01, familiar ob vs. novel ob)

Fig.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.

Fig. 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 day14)

Fig. 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 $\pm$ SEM. ($n=8/\text{group}$, $*p<0.01$, familiar ob vs. novel ob). (B). Representative traces of fEPSP for pre-stimulation baseline, high frequency stimulation (HFS), and application of paclitaxel (10 μ M). (C) Averaged field EPSP data. HFS was given at 21 min, paclitaxel was applied at 41 min and signal was followed for up to 120 min. The application of paclitaxel reduced LTP at CA3-CA1 field of the hippocampus. (D) Summary of changes in fEPSPs

(from 20 to 120 min) shown in C. Data values are expressed as mean $\pm$ SEM. (n=6, * p <0.01, control vs. paclitaxel)

Fig. 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, * $p < 0.05$.

Fig. 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).

Fig. 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 (i.c.v.) 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.

Fig. 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) co-staining 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 day after surgery. (n=5/group, * $p<0.05$).