Modulation of Nerve Injury–induced HDAC4 Cytoplasmic Retention Contributes to Neuropathic Pain in Rats

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ABSTRACT

Background: The histone deacetylases (HDACs) have been implicated in pain hypersensitivity. This study investigated the potential involvement of an HDAC4-related mechanism in the spinal nerve ligation (SNL)-induced nociceptive hypersensitivity.

Methods: The left L5 to L6 spinal nerves of 627 adult male Sprague–Dawley rats were surgically ligated. The withdrawal threshold of hind paws and the abundances, cellular location, and interactions of proteins in the dorsal horn were assayed before and after surgery. The 14-3-3β-targeting small-interfering RNA, a serum- and glucocorticoid-inducible kinase 1 (SGK1) antagonist, or an HDAC inhibitor was spinally injected to elucidate the role of 14-3-3β, SGK1, and HDAC4.

Results: Without affecting the HDAC4 level, SNL provoked SGK1 phosphorylation (mean ± SEM from 0.24 ± 0.02 to 0.78 ± 0.06 at day 7, n = 6), HDAC4 phosphorylation (from 0.38 ± 0.03 to 0.72 ± 0.06 at day 7, n = 6), 14-3-3β expression (from 0.53 ± 0.09 to 0.88 ± 0.09 at day 7, n = 6), cytoplasmic HDAC4 retention (from 1.18 ± 0.16 to 1.92 ± 0.11 at day 7, n = 6), and HDAC4-14-3-3β coupling (approximately 2.4-fold) in the ipsilateral dorsal horn in association with behavioral allodynia. Knockdown of spinal 14-3-3β expression prevented the SNL-provoked HDAC4 retention (from 1.89 ± 0.15 to 1.32 ± 0.08 at day 7, n = 6), HDAC4-14-3-3β coupling (approximately 0.6-fold above SNL 7D), and behavioral allodynia (from 0.16 ± 0.3 to 6 ± 1.78 at day 7, n = 7), but not SGK1 (from 0.78 ± 0.06 to 0.71 ± 0.04 at day 7, n = 6) or HDAC4 (from 0.75 ± 0.15 to 0.68 ± 0.11 at day 7, n = 6) phosphorylation.

Conclusion: Neuropathic pain maintenance involves the spinal SGK1 activation–dependent HDAC4 phosphorylation and its subsequent association with 14-3-3β that promotes cytoplasmic HDAC4 retention in dorsal horn neurons.
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ACCUMULATING evidence has revealed that activity-dependent neural plasticity in the spinal cord underlies pain hypersensitivity[1]; therefore, both clinicians and scientists are highly interested in developing drugs targeted to the pain-associated plasticity to provide medical therapeutics for pain relief.

Intrathecal administration with a class IIa histone deacetylase (HDAC) inhibitor attenuated complete Freund adjuvant–induced inflammation hyperalgesia[2] and deletion of the functional domain of HDAC4, a member of the class IIa HDACs,[3] and increased the response latency to nociceptive stimuli,[4] indicating HDAC4 is a crucial contributor to the spinal machinery underlying heightening of nociception.

What We Already Know about This Topic

- Pain-associated hypersensitivity (allodynia) involves activity-dependent effects on spinal neuroplasticity
- Regulation of histone acetylation by histone deacetylase (HDAC) regulates gene transcription contribute to neuropathic pain

What This Article Tells Us That Is New

- In a rat model of neuropathic pain, HDAC4 phosphorylation led to its cytoplasmic retention due to phosphorylation-dependent interaction with 14-3-3β
- Inhibition of histone deacetylase phosphorylation reduced allodynia and prevented its cytoplasmic translocation, suggesting a novel therapeutic target for neuropathic pain

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Unlike other HDACs that either reside in the nucleus or are mostly in the cytoplasm, class IIa HDACs actively shuttle between the nucleus and cytoplasm.[5] The nucleocytoplasmic shuttling of HDAC4 is primarily regulated through the phosphorylation of its serine residues, that is, phosphorylation prompts HDAC4 export from the nucleus that subsequently recruits 14-3-3 to anchor the resulting complex and thereby restricts HDAC4 location to the cytoplasm.[6] Conversely, dephosphorylation uncouples HDAC4 from 14-3-3, thereby allowing HDAC4 to shuttle into the nucleus.[7] By binding to the phospho-serine-/threonine-containing motifs of a variety of proteins, 14-3-3, a ubiquitous phosphor-binding protein,[8] regulates a wide range of cellular processes including intracellular protein trafficking.[9] Neuropathic injury selectively up-regulated 14-3-3 expression in rat dorsal horn,[10] and 14-3-3β-HDAC4 interactions subsequent to HDAC4 phosphorylation have been linked to diverse physiological/pathological cellular programs.[11] Nevertheless, it remains unclear that whether spinal HDAC4 phosphorylation and the following HDAC4-14-3-3β coupling could participate in the development of nociception hypersensitivity *via* regulating the subcellular HDAC4 distribution.

By phosphorylating the serine residue of neural precursor cell-expressed developmentally down-regulated protein 4-2 (Nedd4-2), serum- and glucocorticoid-inducible kinase 1 (SGK1), a serine/threonine kinase,[12] facilitates the association of Nedd4-2 with 14-3-3.[9] Interestingly, our studies have previously demonstrated that spinal SGK1 crucially participates in the nociception-associated plasticity by regulating serine/threonine phosphorylation–dependent protein trafficking[13] and anchoring.[14] Together, these several observations prompted us to test whether SGK1-dependent HDAC4 phosphorylation, coupling with 14-3-3β, and cytosolic accumulation in dorsal horn neurons contribute to the neuropathic injury–induced nociceptive hypersensitivity (fig. 1).

Materials and Methods

Animal Preparations

The animal procedures in this study were conducted in accordance with the guidelines of the International Association for the Study of Pain[15] and were reviewed and approved by the Institutional Review Board of National Chung-Hsing University, Taichung, Taiwan, Republic of China. A total of 627 adult male Sprague–Dawley rats (180 to 230 g body weight) were used throughout this study. Animal allocation to treatment groups was randomized by a computer with the use of research randomizer, and the sample size of each group was based on our previous experience. There were 119, 294, 14, and 192 rats, respectively, for behavioral test, Western blot, immunohistochemistry, and coprecipitation analyses performed by investigators blinded to treatment groups. There were eight animals that showed neurological deficits after implantation of a catheter and were excluded for statistical analysis. Thus, there were a total of 619 animals used for statistical analysis.

Spinal Nerve Ligation

After anesthesia (isoflurane, induction 5%, maintenance 2%), the left L5 to L6 spinal nerves were carefully isolated and was tightly ligated with 6-0 silk sutures 2 to 5 mm distal to the dorsal root ganglia.[13,14,16] The procedures for the sham operation were identical to the nerve ligation group, excepting the silk sutures were left unligated.

Intrathecal Catheter

An PE-10 silastic tubing was implanted to the lumbar enlargement of the spinal cord. Animals were allowed to recover 3 days after implantation, and those that showed neurological deficits after surgery were sacrificed and excluded from statistical analyses.[17,18]

Behavioral Studies

Tactile sensitivity was assessed by measuring the paw-withdrawal threshold of rats in response to probing with von Frey monofilaments (Stoelting, USA).[19] Animals' tactile thresholds before operation were set equal to 15 g.[13] In some animals, the motor function was assessed using an accelerating rota-rod apparatus (LE8500; Ugo Basile, Italy). For acclimatization, animals were subjected to three training trials at 3- to 4-h intervals on 2 separate days. In training sessions, the rod was set to accelerate from 3 to 30 rpm over a 180-s period. In the test session, the performance times of rats were recorded up to a cutoff time of 180 s. Three measurements tested at intervals of 5 min were averaged in each test.[20]

Western Blotting

The dissected dorsal horn (L4 to L5) sample was homogenized in 25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate with a complete protease inhibitor mixture (Roche, Germany). After incubation on ice (1 h), the lysates were centrifuged (14,000*g*, 20 min, 4°C). The cytoplasmic, nuclear extracts, and membrane protein were prepared by extraction reagent kits (NE-PER and Mem-PER; Thermo Scientific, USA) according to the manufacturer's instructions. Protein concentrations were determined using a bicinchoninic acid assay. In brief, the supernatant was separated on acrylamide gel and transferred to a polyvinylidene difluoride membrane and then incubated (1 h, room temperature) in either rabbit anti-total HDAC4 (tHDAC4, 1:1,000; Genetex, USA), rabbit anti-phosphorylated HDAC4 (pHDAC4, 1:1,000; Genetex), rabbit anti-t14-3-3β (1:1,000; Millipore, USA), rabbit anti-pSGK (1:1,000; Abcam, United Kingdom), rabbit anti-tSGK1 (1:800; Santa Cruz Biotechnology, USA), or mouse antiglyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:20,000; Novus, USA). Blots were washed and incubated (1 h, room temperature) in peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:8,000; Jackson

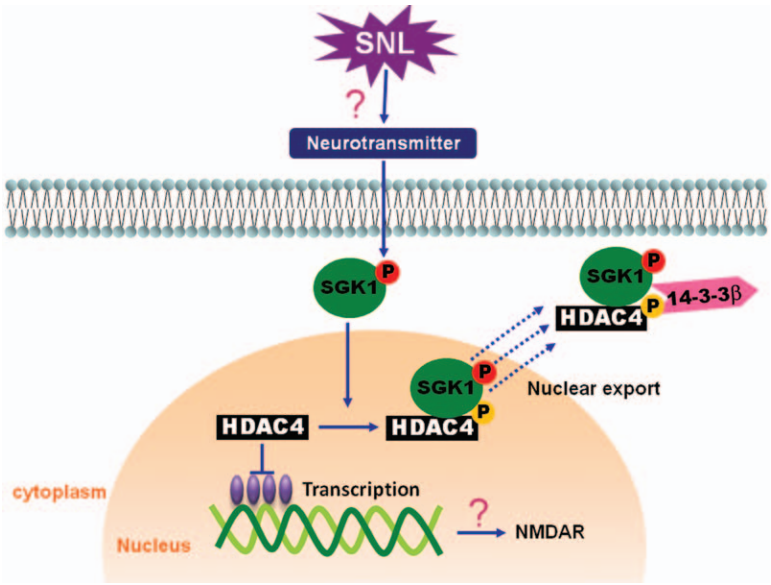

Fig. 1. Schematic diagram showing serum- and glucocorticoid-inducible kinase 1 (SGK1), histone deacetylase (HDAC4), and 14-3-3β contribute to the neuropathic injury–associated nociceptive hypersensitivity. Possibly through regulating neurotransmitter release, spinal nerve ligation (SNL) activates SGK1 that provokes HDAC4 phosphorylation and the subsequent HDAC4-14-3-3β coupling, which restricts HDAC4 location to the cytoplasm of dorsal horn neurons. Reduced inhibition of HDAC4 on DNA transcription might up-regulate plasticity-associated protein, such as the *N*-methyl-D-aspartate receptor (NMDAR).

ImmunoResearch, USA) or goat anti-mouse immunoglobulin G (1:8,000; Jackson ImmunoResearch). Protein bands were visualized by using an enhanced chemiluminescence detection kit (ECL Plus; Millipore), and, then, densitometric analysis of the Western blot membranes was performed with Science Lab 2003 (Fuji, Japan).

Coprecipitation

Rabbit polyclonal antibody against tHDAC4, t14-3-3β, and tSGK1 was incubated (overnight, 4°C) with the extraction of the dorsal horn samples. The 1:1 slurry protein agarose suspension (Millipore) was added into that immunocomplex of protein, and the mixture was incubated (2 to 3 h, 4°C). Agarose beads were washed once with 1% (v/v) Triton X-100 in an immunoprecipitation buffer (50 mM Tris-Cl, pH 7.4, 5 mM EDTA, and 0.02% [w/v] sodium azide), twice with 1% (v/v) Triton X-100 in an immunoprecipitation buffer plus 300 mM NaCl, and thrice with an immunoprecipitation buffer only. Binding proteins were eluted with sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer at 95°C. Proteins were separated with the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes electrophoretically, and detected by using rabbit anti-tHDAC4 (1:1,000; Genetex), rabbit anti-pHDAC4 (1:1,000; GeneTex), rabbit anti-t14-3-3β (1:1,000; Millipore), rabbit anti-pSGK (1:1,000; Abcam), and rabbit anti-tSGK1 (1:800; Santa Cruz Biotechnology).

Immunofluorescence

After perfusion (100 ml phosphate-buffered saline followed by 300 ml paraformaldehyde, 4%; pH 7.4), the spinal cord samples were harvested, postfixed (4°C for 4 h), and cryoprotected in sucrose solution (30%) overnight. For double-labeling analyses investigating interactions between HDAC4 and neuronal/glial/microglia markers, the spinal sections were incubated with a mixture of rabbit anti-tHDAC4 (1:200; GeneTex) and mouse monoclonal antineuronal nuclear antigen (NeuN, a neuronal marker, 1:500; Millipore Bioscience Research Reagents), mouse antiglial fibrillary acidic protein (a marker of astroglial cells; 1:1,000; Millipore), or mouse anti-integrin αM (OX-42, a marker of microglia; 1:500; Santa Cruz Biotechnology) overnight (4°C). Then the sections were then incubated (1 h, 37°C) with Alexa Fluor 488 (1:1,500) and Alexa Fluor 594 (1:1,500; Invitrogen). When examining the interaction between HDAC4, 14-3-3β, and SGK1, the specific antibodies were mixed with 10X reaction buffer (Mix-n-Stain; Biotium, USA) with the antibody solution at a ratio of 1:10. Then, the solution was transferred to the viral containing dye (CF; Biotium) and incubated in the dark (30 min, at room temperature). The spinal cord sections were sequentially incubated (overnight, 4°C) with dilute solutions, that is, rabbit-pHDAC4 (200X; GeneTex), rabbit anti-t14-3-3β (×1000; Millipore), and rabbit anti-pSGK (×1,000; Abcam), and were washed five times in between each incubation.

Small-interfering RNA

The 19 nucleotide duplexes of the small-interfering RNAs (siRNAs) for 14-3-3β were 5'-GAAAUACAAUUCUGUUGUA-3' and missense nucleotides were 5'-UACAACAGAAUUGUAUUUC-3'. The missense or siRNA with polyethyleneimine (10 μl, Al 25 kDa; Sigma–Aldrich,

Germany)-based gene delivery system was intrathecally into the dorsal subarachnoid space (L4 to L5) of animals through the implanted catheter (daily for 4 days).

Drugs Application

GSK-650394 (a SGK1 inhibitor; 100 nM, 10 μl; Tocris Bioscience, USA) or LMK235 (an HDAC4 inhibitor; 10, 30, and 100 nM, 10 μl; Tocris Bioscience) was administered intrathecally by bolus injection. A vehicle solution of a volume identical to that of the tested agents was dispensed to serve as a control.

Data Analysis

All the data in this study were analyzed using SigmaPlot 10.0 (Systat Software, U.S.A.) or Prism 6.0 (GraphPad, U.S.A.) and are expressed as the mean ± SEM. Paired two-tailed Student *t* test was used to compare the means between groups. One-way or two-way ANOVAs were used to assess the changes in values for serial measurements over time, and *post hoc* Tukey tests were used to compare the means of groups. Significance was set at *P* value less than 0.05.

Results

Spinal Nerve Ligation Induced Allodynia-associated Cytoplasmic HDAC4 Retention

Western blot analysis of dorsal horn samples (L4 to L5) demonstrated that the amount of the total HDAC4 protein (fig. 2A; tHDAC4) was uniformly expressed at days 1, 3, 7, 14, and 21 after operation in the ipsilateral (I) and contralateral (C) dorsal horn of both the spinal nerve ligation (SNL; all $P > 0.05$ compared with day 1, n = 6) and sham operation (Sham; all $P > 0.05$ compared with day 1, n = 6) groups. Nevertheless, when compared with the sham operation, SNL significantly increased the cytoplasm/nuclear ratio of tHDAC4 by increasing the abundance of the cytoplasmic but decreasing that of the nuclear HDAC4 in the ipsilateral (fig. 2B; $P < 0.001$, $P < 0.001$, $P = 0.005$, and $P = 0.006$, respectively compared with day 1, n = 6), but not the contralateral (all $P > 0.05$ compared with day 1, n = 6), dorsal horn at days 3, 7, 14, and 21 after operation, implying SNL-provoked cytoplasmic HDAC4 accumulation in the ipsilateral dorsal horn. The SNL-provoked HDAC4 retention was parallel to the time course of decrements in withdrawal latency, which occurred at days 3, 7, 14, and 21 after operation (fig. 2C; all $P < 0.001$ compared with day 1, n = 7). These results suggest that SNL-induced behavioral allodynia is accompanied with HDAC4 translocation in the dorsal horn.

Spinal HDAC4 Was Localized in Dorsal Horn Neurons

Double-staining immunohistochemistry images revealed that most of the HDAC4 immunofluorescence (fig. 2D; I tHDAC4) occurred coincidently with NeuN (II, a neuronal marker), instead of OX-42 (III, a microglial marker) or glial fibrillary acidic protein (IV, an astrocyte marker) immunofluorescence, in the ipsilateral dorsal horn (L4 to L5) dissected at day 7 after SNL, suggesting that the neuropathic injury–associated spinal HDAC4 was present in the dorsal horn neurons but rather than in microglia or astrocytes.

SNL Induced HDAC4 Phosphorylation and Coupling with 14-3-3β

Spinal nerve ligation significantly increased the abundance of the phosphorylated HDAC4 (fig. 3A; pHDAC4) in the ipsilateral (I; $P = 0.003$, $P = 0.004$, $P = 0.006$, and $P = 0.009$, respectively, compared with day 1, n = 6), but not the contralateral (C; all $P > 0.05$ compared with day 1, n = 6), dorsal horn sample at days 3, 7, 14, and 21 after operation. SNL also up-regulated the expression of total 14-3-3β (fig. 3B; t14-3-3β) selectively in the ipsilateral dorsal horn at these time points ($P = 0.005$, $P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively, compared with day 1, n = 6). However, the sham operation exhibited no effect in the abundance of pHDAC or t14-3-3β at days 3, 7, 14, and 21 after operation (fig. 3, A and B; all $P > 0.05$ compared with day 1, n = 6). When compared with the preoperation control (fig. 3C; day 1), SNL increased the amounts of tHDAC4-bound pHDAC4 and t14-3-3β in the tHDAC4-recognized precipitates at days 3 and 7 after operation (approximately 1.8- and 2.4-fold above day 1 in pHDAC4 and 1.4- and 1.6-fold above day 1 in t14-3-3β, respectively). The amount of t14-3-3β-bound pHDAC4 also was enhanced at the same time points in the 14-3-3β-recognized precipitates (approximately 2.0- and 2.8-fold above day 1). Nevertheless, the abundance of GAPDH in the preprecipitated homogenates obtained at these time points remained at a relative constant level. At day 7 after operation, SNL notably enhanced the immunofluorescence of pHDAC4 (fig. 4A; I and IV pHDAC4) and total 14-3-3β (II and V t14-3-3β) in the ipsilateral (IPSI) compared with the contralateral dorsal horn (CONTRA I and VII pHDAC4; II and VIII t14-3-3β). Merged images showed that most of the pHDAC4 immunoreactivity was coincident with that of 14-3-3β (III, VI, and VIa) in the ipsilateral dorsal horn. Statistical analysis revealed that SNL significantly enhanced the numbers of pHDAC4-, t14-3-3β-, and pHDAC4-t14-3-3β double-labeled neurons in the ipsilateral compared with the contralateral dorsal horn (fig. 4B; all $P < 0.01$ compared with CONTRA, n = 7). Together, these results showed that SNL provoked HDAC4 phosphorylation, 14-3-3β expression, HDAC4-14-3-3β coupling/colocalization, and HDAC4 translocation in dorsal horn neurons that have a temporal correlation with behavioral allodynia.

14-3-3β Knockdown Ameliorated Allodynia, HDAC4 Translocation, and HDAC4-14-3-3β Coupling

Although exhibiting no effect on the level of GAPDH, the administration with 14-3-3β messenger RNA (mRNA)-targeting siRNA (fig. 5A; 14-3-3β messenger RNA-targeting small interfering RNA (14-3-3β RNAi); 10 μl, $P = 0.243$,

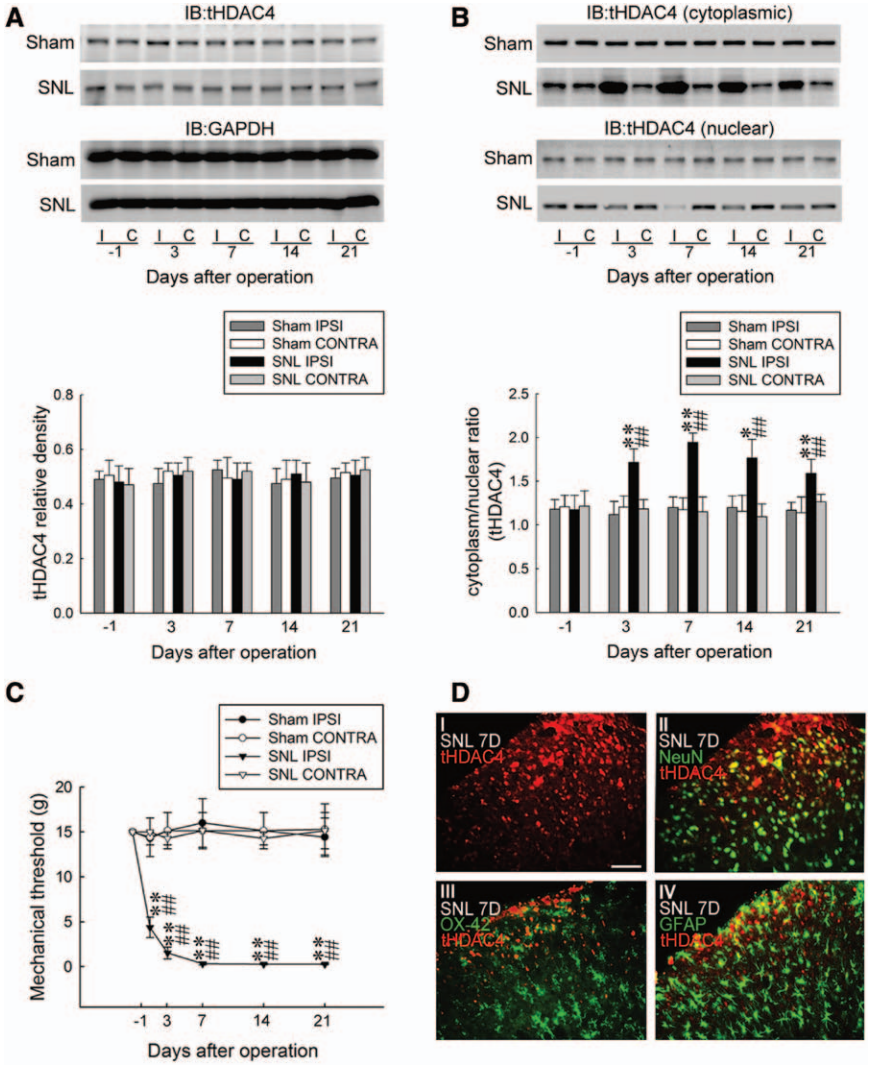

Fig. 2. Neuropathic injury induces behavioral allodynia and cytoplasmic histone deacetylase (HDAC4) retention in dorsal horn neurons. (*A*) Representative Western blots and statistical analysis showing total HDAC4 (tHDAC4) were uniformly expressed at days 1, 3, 7, 14, and 21 after operation in the ipsilateral (I, IPSI) and contralateral (C, CONTRA) dorsal horn of the spinal nerve ligation (SNL) and sham operation (Sham) groups ($P > 0.05$ compared with day 1, n = 6). (*B*) SNL statistically increased the tHDAC4 abundance in the cytoplasmic fraction but decreased that in the nuclear fractions of the ipsilateral dorsal horn samples at days 3, 7, 14, and 21 after operation that resulted in an increased cytoplasmic/nuclear ratio of tHDAC4 (*$P < 0.05$, **$P < 0.01$ compared with Sham IPSI; ##$P < 0.01$ compared with day 1, n = 6). (*C*) Von Frey test showing SNL significantly decreased the mechanical threshold of the ipsilateral hind paw at days 1, 3, 7, 14, and 21 after operation (**$P < 0.01$ compared with Sham IPSI; ##$P < 0.01$ compared with day 1, n = 7). (*D*) Immunohistochemistry images showing the tHDAC4 immunoreactivity (I; *red*) was colabeled (*yellow*) with NeuN (II; *green*, a marker for neurons), but not OX-42 (III; *green*, a marker for microglia) or glial fibrillary acidic protein (IV GFAP; *green*, a marker for astrocytes), in the ipsilateral dorsal horn of spinal cord sections obtained at day 7 after operation (SNL7D). *Scale bar* = 50 μm, thickness = 50 μm. Each of these images was replicated in seven sample preparations with similar results each time. GADPH = glyceraldehyde 3-phosphate dehydrogenase; IB = immunoblotting.

$P < 0.001$, and $P < 0.001$ in 1, 3, and 5 μg, respectively, compared with naive, n = 6), but rather than the administration with missense siRNA (14-3-3β MS; 5 μg, 10 μl), administration with polyethylenimine (a transfection reagent; 10 μl), or implantation of intrathecal catheter (it), dose dependently decreased the abundance of t14-3-3β in the dorsal horn samples when compared with the control levels in naive animals (Naive; all $P > 0.05$ compared with naive, n = 6), indicating specific siRNA sufficiently knocked-down spinal 14-3-3β expression. No statistical difference was evidenced in the performance time of the rota-rod test between the naive and polyethylenimine-, missense siRNA-, or 14-3-3β mRNA-targeting siRNA (3 μg, 10 μl)-treated animals (fig. 5B; all $P > 0.05$ compared with day 0, n = 7), indicating that our knockdown procedures resulted in no motor deficits in animals. All the treatments, including catheter implantation and antisense or missense siRNA injection, exhibited no difference in the withdrawal

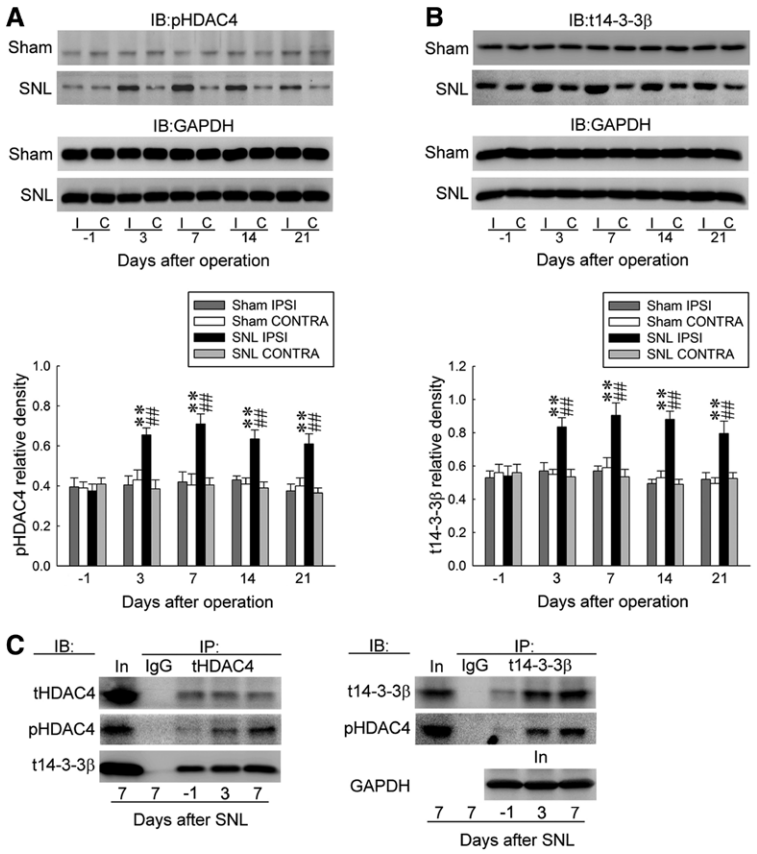

Fig. 3. Neuropathic injury provokes spinal phosphorylated histone deacetylase (pHDAC4) and 14-3-3β expressions and HDAC4-14-3-3β coupling. (*A* and *B*) Representative Western blots and statistical analysis showing, when compared with the sham operation (Sham), spinal nerve ligation (SNL) statistically up-regulated the level of pHDAC4 and total 14-3-3β (t14-3-3β) at days 3, 7, 14, and 21 after operation in the ipsilateral (I, IPSI) but not the contralateral (*C*, CONTRA) dorsal horn (**P < 0.01 compared with the Sham IPSI; ##P < 0.01 compared with day 1, n = 6). (*C*) Coimmunoprecipitation analysis showing SNL enhanced the tHDAC4-bound pHDAC4 and t14-3-3β- and t14-3-3β-bound pHDAC4 in the dorsal horn samples at days 3 and 7 after operation. In both cases, no detectable immunoreactivity was labeled by antibodies in the control IgG-recognized precipitation. GADPH = glyceraldehyde 3-phosphate dehydrogenase; IB = immunoblotting; IgG = immunoglobulin G; In = input control; IP = immunoprecipitation.

threshold of von Frey test measured in sham-operated animals (fig. 5C; Sham; all P > 0.05 compared with Sham, n = 7). In contrast, the spinal administration with 14-3-3β mRNA-targeting siRNA (fig. 5D; SNL + it + 14-3-3β RNAi; P = 0.001 and P < 0.001, respectively, compared with SNL, n = 7), but not the administration with missense siRNA (SNL + it + 14-3-3β MS) or catheter implantation alone (SNL + it), partially ameliorated behavioral allodynia as evidenced by a significant increase in the withdrawal threshold at days 5 and 7 after operation compared with animals that received SNL only (SNL; all P > 0.05 compared with SNL, n = 7), indicating that loss of dorsal horn 14-3-3β alleviated SNL-associated allodynia. Although both treatments failed to affect the SNL-enhanced pHDAC4 level (fig. 5E; all P > 0.05 compared with SNL 7D, n = 6), daily administration with 14-3-3β mRNA-targeting siRNA (fig. 5F; SNL 7D + 14-3-3β RNAi; P = 0.015 compared with SNL 7D, n = 6), but rather than the missense siRNA (SNL 7D + 14-3-3β MS; P > 0.05 compared with SNL 7D, n = 6), attenuated the SNL-provoked cytoplasmic HDAC4 retention in the ipsilateral dorsal horn when compared with the SNL group. Administering with 14-3-3β mRNA-targeting siRNA reduced the amount of tHDAC4-bound t14-3-3β in the ipsilateral dorsal horn measured at day 7 after nerve ligation (approximately 0.6-fold above SNL 7D) when compared with animals that received no siRNA (SNL 7D; approximately 1.9-fold above Sham 7D). Nevertheless, it failed to affect SNL-enhanced abundance of tHDAC4-bound pHDAC4 (approximately 1.1-fold above SNL 7D; fig. 5G). Together with the above results, these data revealed that without affecting HDAC4 phosphorylation, the 14-3-3β knockdown ameliorated SNL-induced allodynia as well as the HDAC4-14-3-3β coupling and cytoplasmic HDAC4 accumulation.

GSK-650394 Prevented Allodynia, HDAC4-14-3-3β Coupling, and HDAC4 Phosphorylation/Translocation

A bolus injection of GSK-650394 (fig. 6A; SNL 7D + GSK; an SGK1 inhibitor; 100 nM, 10 μl: P < 0.001, P < 0.001, P = 0.001, P = 0.01, and P = 0.01, respectively, compared with hour 0, n = 7), but not the vehicle solution (SNL 7D

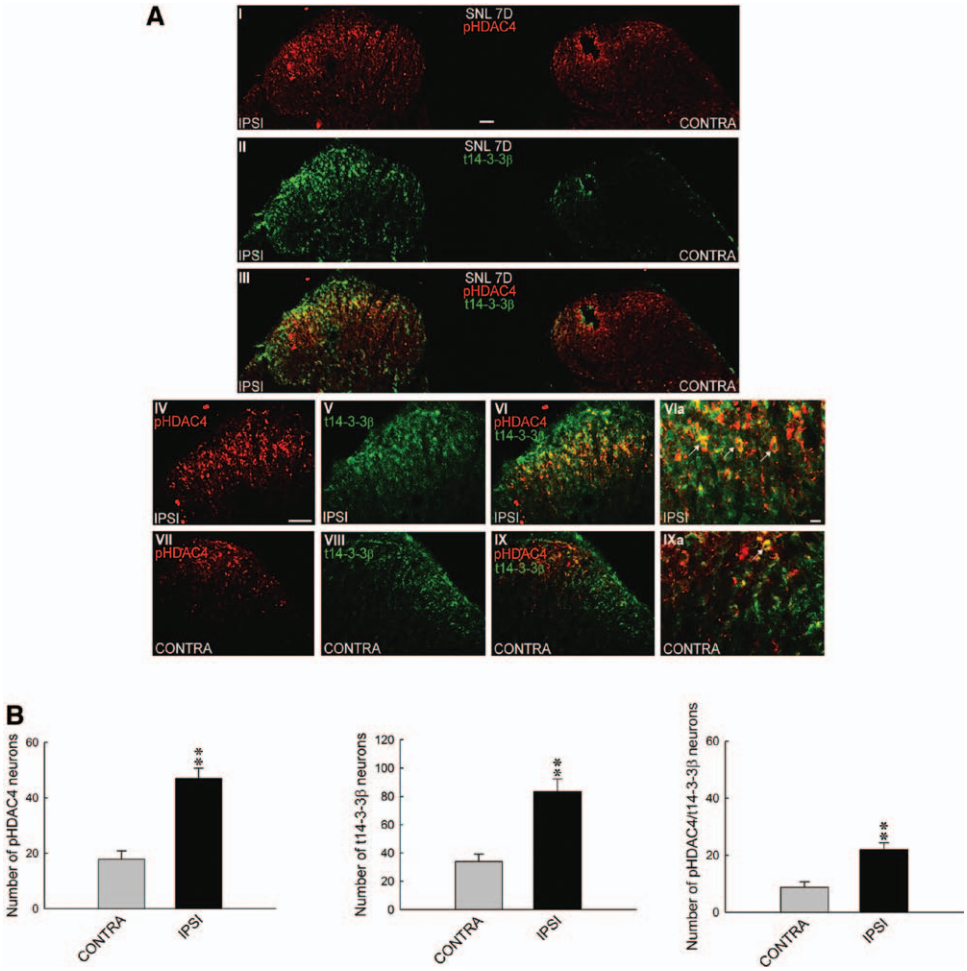

Fig. 4. Neuropathic injury up-regulates colocalized phosphorylated histone deacetylase (pHDAC4) and t14-3-3β expressions in the ipsilateral dorsal horn. (*A*) Immunohistochemical images showing that spinal nerve ligation (SNL) enhanced the immunoreactivity of pHDAC4 (I and IV, *red*) and total 14-3-3β (t14-3-3β; II and V, *green*) in the ipsilateral (IPSI) compared with the contralateral (CONTRA) dorsal horn (I, VII and II, VIII, respectively) of the spinal sections obtained at day 7 after operation (7D). Overlay images and the count of double-labeled neurons showing the enhanced pHDAC4 fluorescence (IV) costained with t14-3-3β immunoreactivity (V) in the ipsilateral (III, VI, and VIa, *yellow*), but not in the contralateral (III, IX, and IXa), dorsal horn. *Scale bar* = 50 μm, thickness = 50 μm. Each of these immunofluorescence images was replicated in seven sample preparations with similar results each time. (*B*) Statistical analysis showing SNL enhanced the count of pHDAC4-positive, t14-3-3β-positive, and double-labeled neurons in the ipsilateral dorsal horn of the spinal sections obtained at day 7 after operation (**P < 0.01 compared with the CONTRA, n = 7).

+ Veh; all P > 0.05 compared with hour 0, n = 7), significantly alleviated SNL-induced allodynia at hours 1, 2, 3, 4, and 5 after administration on day 7 after SNL when compared with animals subjected to SNL only. This result indicates that antagonism of spinal SGK1 alleviated neuropathic injury–established nociception enhancement. Although the intrathecal GSK-650394 injection significantly reversed the increases in the pHDAC4 amount (fig. 6B; SNL 7D + GSK; P = 0.015 compared with SNL 7D, n = 6) and the cytoplasmic/nuclear ratio of tHDAC4 (fig. 6C; SNL 7D + GSK; P < 0.001 compared with SNL 7D, n = 6), it failed to affect the SNL-enhanced 14-3-3β expression (fig. 6D; SNL 7D + GSK; P > 0.05 compared with SNL 7D, n = 6) in the ipsilateral dorsal horn. The SNL-enhanced amounts of tSGK1-bound pSGK1, pHDAC4, and t14-3-3β (approximately 1.8-, 2.4- and 2.0-fold above Sham 7D) as well as tHDAC4-bound pSGK1, pHDAC4, and t14-3-3β (approximately 2.0-, 1.8-, and 2.2-fold above Sham 7D) in the ipsilateral dorsal horn were reduced by the spinal GSK-650394 injection (approximately 0.6-, 0.2- and 0.4-fold above SNL 7D in tSGK1 and 0.7-, 0.5-, and 0.4-fold above SNL 7D in tHDAC4, respectively). Although daily administration with 14-3-3β mRNA-targeting siRNA (SNL 7D + 14-3-3β RNAi) decreased the tSGK1- and f4-bound 14-3-3β (approximately 0.2- and 0.3-fold above SNL 7D, respectively), it failed to affected the abundance of tSGK1-bound pSGK1 and pHDAC4 (approximately 1.4- and 1.4-fold above Sham 7D in pSGK1 and 2.2- and 2.3-fold above SNL 7D in pHDAC4, respectively)

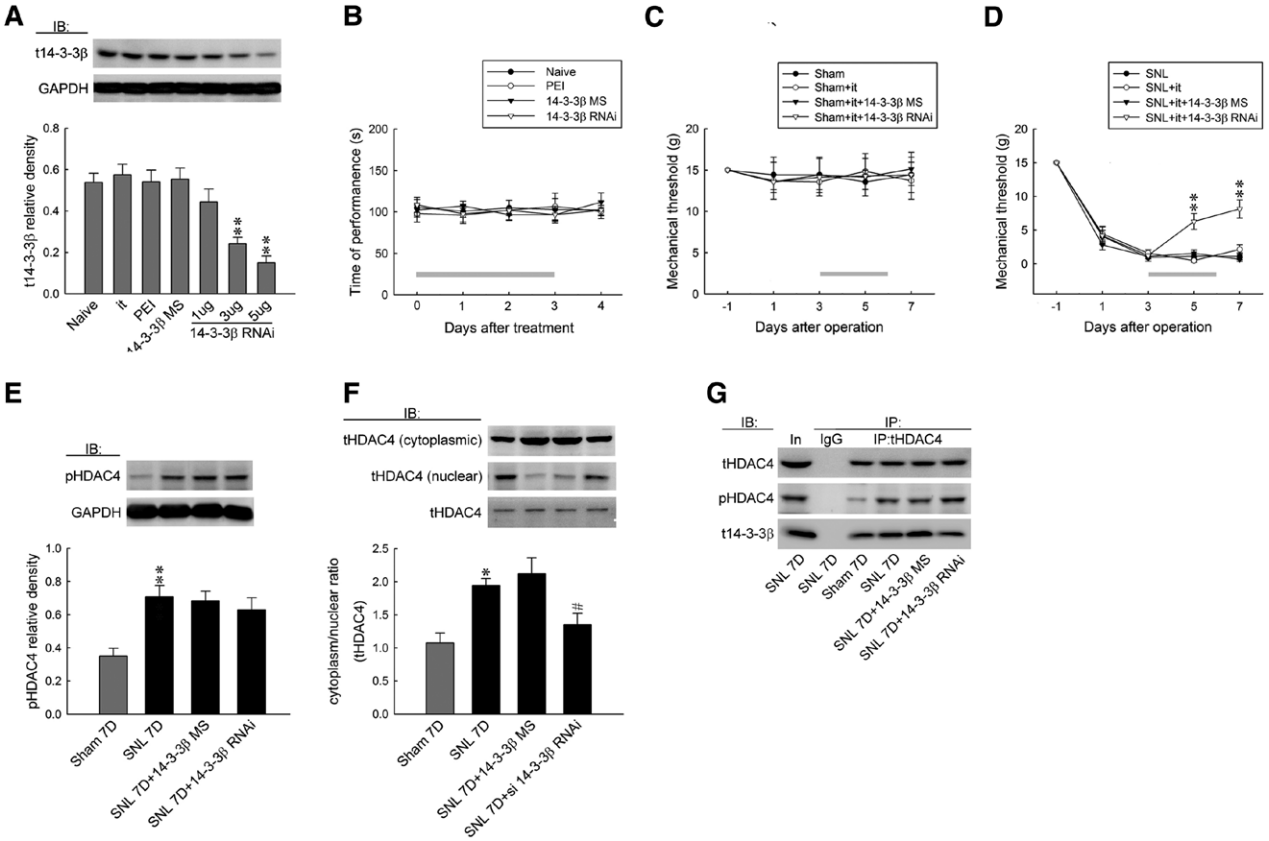

Fig. 5. 14-3-3β small-interfering RNA (siRNA) prevents neuropathic injury–associated allodynia, histone deacetylase (HDAC4) translocation, and HDAC4-14-3-3β coupling. (*A*) Representative Western blot and statistical analysis showing administration with 14-3-3β messenger RNA-targeting small interfering RNA (14-3-3β RNAi), but not intrathecal catheter implantation (it), administration with polyethylenimine (PEI; a transfection reagent; 10 μl), or missense siRNA (14-3-3β MS, 5 μg, 10 μl), dose dependently decreased the level of total 14-3-3β (t14-3-3β) in the dorsal horn (***P* < 0.01 compared with the Naive, n = 6). (*B*) Administration with PEI, missense siRNA, and 14-3-3β mRNA-targeting siRNA (3 μg, 10 μl) failed to affect the performance times of rata-rod test at days 0, 1, 2, 3, and 4 after treatment compared with the naive animals (*P* > 0.05 compared with naive, n = 7). (*C* and *D*) Although treatments failed to produce statistical differences in sham-operated animals (Sham; *P* > 0.05 compared with Sham, n = 7), 14-3-3β RNA-targeting siRNA significantly increased the withdrawal threshold in spinal nerve ligation (SNL) animals at days 5 and 7 after operation (***P* < 0.01 compared with the SNL, n = 7). (*B–D*) The *gray bar* at the bottom indicates the duration of administration. (*E* and *F*) Representative Western blot and statistical analysis showing while 14-3-3β mRNA-targeting siRNA failed to affect the pHDAC4 expression, it significantly decreased the SNL-enhanced cytoplasmic/nuclear tHDAC4 ratio (**P* < 0.05, ***P* < 0.01 compared with Sham 7D, #*P* < 0.05 compared with SNL 7D, n = 6). (*G*) 14-3-3β mRNA-targeting siRNA reversed the SNL-increased tHDAC4-t14-3-3β but rather than tHDAC4-pHDAC4 coprecipitation. No detectable immunoreactivity was labeled by antibodies in the control IgG-recognized precipitation. GADPH = glyceraldehyde 3-phosphate dehydrogenase; IB = immunoblotting; IgG = immunoglobulin G; In = input control; IP = immunoprecipitation.

or tHDAC4-bound pSGK1 and pHDAC4 (approximately 1.1- and 1.0-fold above SNL 7D in pSGK1 and 1.0- and 0.9-fold above SNL 7D in pHDAC4, respectively) measured on day 7 postoperation (fig. 6E). Moreover, when compared with sham operation, SNL provoked dorsal horn SGK1 phosphorylation as evidenced by significantly increased amounts of phosphorylated SGK1 (fig. 6F; pSGK1; *P* < 0.001 compared with Sham 7D, n = 6) in the ipsilateral dorsal horn on day 7 after operation. In contrast to intrathecal GSK-650394 injection that effectively reversed the SNL-enhanced amount of pSGK1 (SNL 7D + GSK; *P* = 0.002 compared with SNL 7D, n = 6), treatments including the spinal injection of the vehicle solution (SNL 7D + Veh), the daily administration with 14-3-3β mRNA-targeting siRNA and missense siRNA (SNL 7D + 14-3-3β MS) failed to affect the SNL-associated increase in pSGK1 level (all *P* > 0.05 compared with SNL 7D, n = 6), indicating that the SNL-induced SGK1 phosphorylation and SGK1-HDAC4 interactions were not affected by focal knockdown of spinal 14-3-3β expression.

14-3-3β Knockdown Failed to Affect SGK1-HDAC4 Colocalization

When compared with sham-operated animals (fig. 7A; Sham I and II), SNL notably enhanced the immunoreactivity of

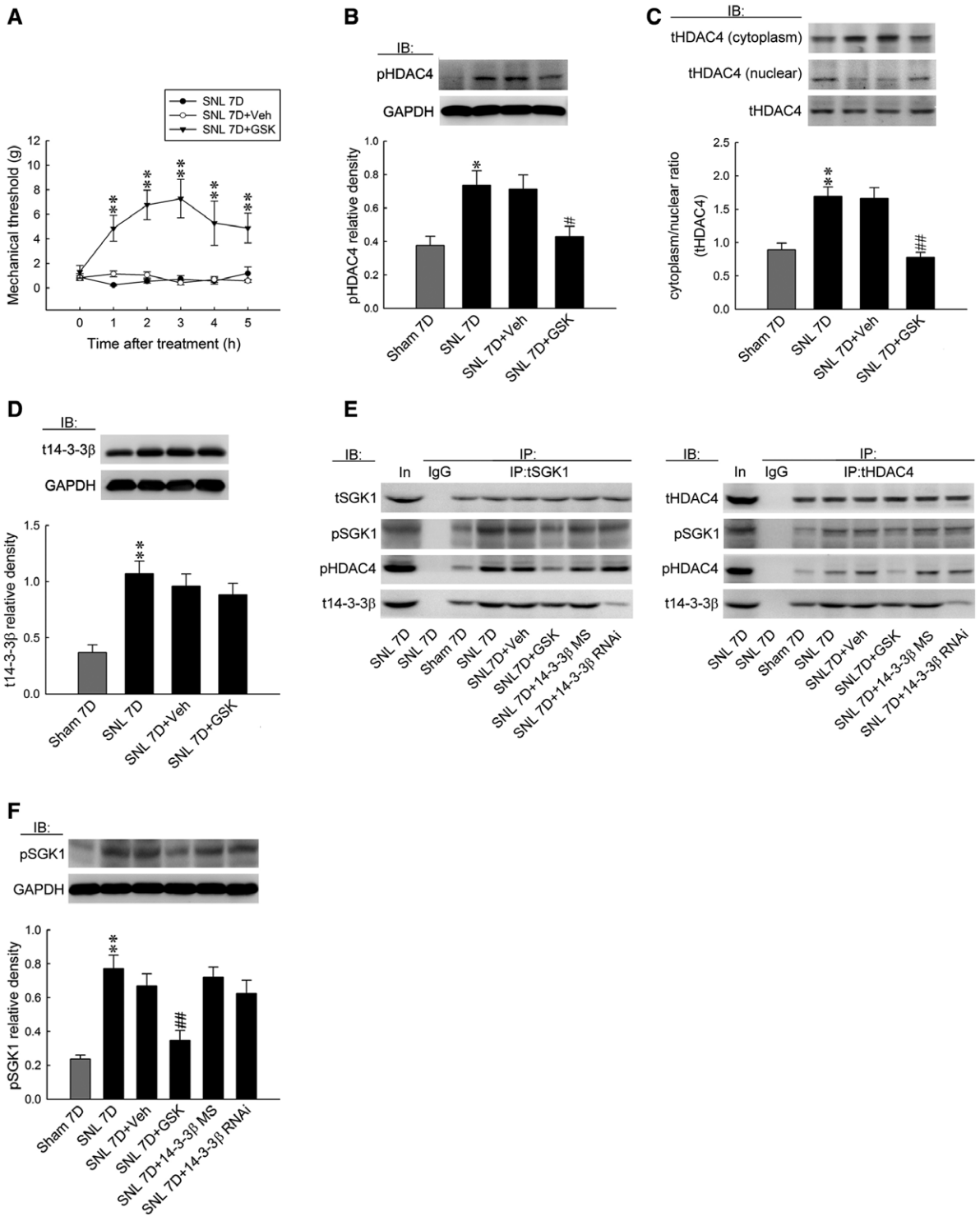

Fig. 6. Neuropathic injury provokes serum- and glucocorticoid-inducible kinase 1 (SGK1)-dependent histone deacetylase (HDAC4) phosphorylation and HDAC4-14-3-3β association in dorsal horn neurons. (*A*) At day 7 (7D) after spinal nerve ligation (SNL), administration with GSK-650394 (an SGK1 inhibitor; 30 nM, 10 μl; SNL 7D + GSK) increased the withdrawal threshold of the ipsilateral hind paw at hours 1, 2, 3, 4, and 5 after treatment (***P* < 0.01 compared with the hour 0, n = 7). (*B–D*) Representative Western blot and statistical analysis showing, when compared with the sham operation (Sham 7D), that SNL enhanced the amount of phosphorylated HDAC4 (*B*, pHDAC4), the cytoplasmic/nuclear ratio of total HDAC4 (*C*, tHDAC4), and the amount of total 14-3-3β (*D*, t14-3-3β) in the dorsal horn (SNL 7D). Bolus of GSK-650394 decreased the SNL-enhanced pHDAC4 and cytoplasmic/nuclear ratio of tHDAC4 but exhibited no effect on the SNL-enhanced t14-3-3β abundance (**P* < 0.05, ***P* < 0.01 compared with Sham 7D; #*P* < 0.05, ##*P* < 0.01 compared with the SNL 7D, n = 6). (*E*) Coprecipitation analysis showing SNL notably increased the amounts of tSGK1-bound pSGK1, pHDAC4, and t14-3-3β and tHDAC4-bound pSGK1, pHDAC4 and

pSGK1 and pHDAC4 in the ipsilateral dorsal horn (IV and V). In addition, pSGK1 and pHDAC4 were largely shown to colocalize in the merged image (VI and VIa). Intrathecal GSK-650394 injection markedly reversed the SNL-enhanced pSGK1, pHDAC4, and colocalized immunoreactivity in the dorsal horn (VII, VIII, IX, and IXa), whereas daily 14-3-3β mRNA-targeting siRNA (X, XI, XII, and XIIa) had no effect. Statistical analysis revealed, when compared with sham operation, that SNL significantly enhanced the counts of pSGK1-, pHDAC4-, and pSGK1-pHDAC4 double-labeled neurons in the ipsilateral dorsal horn (fig. 7B; all $P < 0.001$ compared with Sham 7D, n = 6) that were reversed by administering with a bolus of GSK-650394 ($P = 0.002$, $P < 0.001$, and $P < 0.001$, respectively, compared with SNL 7D, n = 6) but rather than daily 14-3-3β mRNA-targeting siRNA (all $P > 0.05$ compared with SNL 7D, n = 6). These data indicated that the SNL-provoked SGK1 and HDAC4 phosphorylation and SGK1-HDAC4 colocalization were not affected by focal knockdown of spinal 14-3-3β expression.

LMK235 Prevented Allodynia but Not SGK1 Phosphorylation, HDAC4 Phosphorylation/Translocation, and 14-3-3β Expression

On day 7 after SNL, a bolus injection of LMK235 (fig. 8A; SNL 7D + LMK235; an HDAC4 inhibitor; 10 μl; $P = 0.456$, $P = 0.002$, $P = 0.005$, and $P = 0.112$; $P = 0.005$, $P < 0.001$, $P = 0.001$, and $P < 0.001$; all $P < 0.001$ in 10, 30, and 100 nM, respectively, compared with hour 0, n = 7), but not the vehicle solution (SNL 7D + Veh; all $P > 0.05$ compared with hour 0, n = 7), dose dependently alleviated SNL-induced allodynia at hours 2, 3, 4, and 5 after administration when compared with animals subjected to SNL only, indicating that inhibition of HDAC4 activity can alleviate neuropathic injury–established nociception enhancement. Nevertheless, we observe that intrathecal LMK235 injection (fig. 8, B and C; SNL 7D + LMK235; 100 nM, 10 μl) exhibited no effect on the SNL-enhanced t14-3-3β, pSGK1, pHDAC4 expressions and cytoplasmic/nuclear ratio of tHDAC4 (all $P > 0.05$ compared with SNL 7D, n = 6), indicating that the inhibition of HDAC4 activity by LMK235 might ameliorate SNL-induced allodynia through pathways other than the spinal SGK1/HDAC4/14-3-3β cascade.

Fig. 6. (*Continued*) t14-3-3β that were all significantly reduced by GSK-650394. Although administration with 14-3-3β messenger RNA-targeting small-interfering RNA (siRNA; SNL 7D + 14-3-3β RNAi) decreased the SNL-enhanced amount of tSGK1- and tHDAC4-bound 14-3-3β, it failed to affected that of tSGK1-bound pSGK1 and pHDAC4 or tHDAC4-bound pSGK1 and pHDAC4. No detectable immunoreactivity was labeled by antibodies in the control IgG-recognized precipitates in both cases. (*F*) Representative Western blot and statistical analysis showing administration with GSK-650394, but not the 14-3-3β mRNA-targeting siRNA, decreased the SNL-enhanced abundance of pSGK1 (**$P < 0.01$ compared with the Sham 7D, ##$P < 0.01$ compared with the SNL 7D, n = 6). GADPH = glyceraldehyde 3-phosphate dehydrogenase; IB = immunoblotting; IgG = immunoglobulin G; In = input control; IP = immunoprecipitation; Veh = vehicle solution.

Discussion

Results in this study demonstrated that neuropathic injury–associated nociception hypersensitivity involves spinal SGK1 activation–dependent HDAC4 phosphorylation and the subsequent HDAC4-14-3-3β coupling, which restricts HDAC4 location to the cytoplasm of dorsal horn neurons (fig. 1). We speculate that cytoplasmic HDAC4 retention, which reduces the amount of nucleus-bound HDAC4, could alter nociception-associated gene expression *via* increasing the histone acetylation–dependent chromatin remodeling. Although intrathecally administrated animals with an HDAC4 inhibitor effectively prevented SNL-induced allodynia, it failed to affect the associated HDAC4 phosphorylation. Although mechanisms underlying such a discrepancy are unclear, we suggest that the SGK1-dependent HDAC4 phosphorylation/translocation could contribute to the SNL-enhanced nociception through pathways other than the traditional HDAC4-associated epigenetic modulation, which increases histone acetylation to regulate chromatin remodeling. Our speculation was based on crystallography studies documented HDAC4 has three serine residues at amino acid 246, 467, and 632 that can be phosphorylated and a regulatory zinc-binding domain at amino acid 648 to 675 that allows binding to the transcriptional corepressor.[21] Several HDAC inhibitors have been shown to act as a chelator for the zinc ion in the active site to hamper the association of HDAC with corepressors that impedes HDAC-mediated epigenic modification,[22] and administration of HDAC4 inhibitors could have little effect on HDAC4 phosphorylation. Our proposal was supported by a study showing that class IIa HDAC inhibition, MC1568 or TMP269, does not prevent agonist-induced HDAC4 phosphorylation and nuclear extrusion of HDAC4 in cultured cells.[23] Nevertheless, further experiments are needed to clarify the detailed mechanism.

In the current study, GSK-650394 administration alleviated SNL-induced allodynia as early as 1 h postinjection. This latency does not seem long enough for the recognized epigenetic expression of nociception-associated genes. It worth notice that SGK1 was demonstrated to phosphorylate Nedd4-2 and thus facilitate 14-3-3-Nedd4-2 coupling to regulate membrane trafficking of channel proteins in cultured cells.[9] HDAC4 phosphorylation and subsequent coupling with 14-3-3β were also shown to regulate nucleocytoplasmic HDAC4 shuttling.[7,11] Our previous study demonstrated SGK1 contributes to postinflammatory hyperalgesia by regulating the membrane trafficking of specific glutamatergic receptors, a pivotal spinal machinery underlying nociception hypersensitivity.[13,18] Further experiments should be conducted to elucidate whether subcellular trafficking of glutamatergic receptors lies downstream of SGK1/pHDAC4/HDAC4-14-3-3β cascade to mediate neuropathic pain.

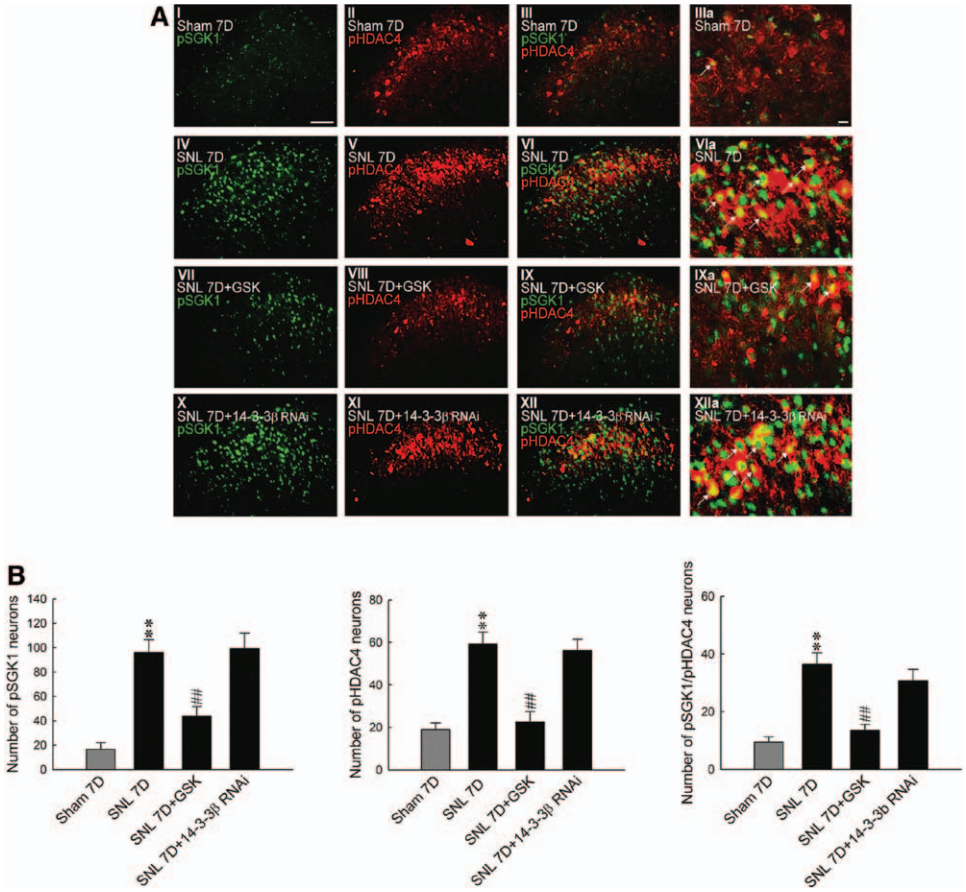

Fig. 7. GSK-650394, but not 14-3-3β small-interfering RNA (siRNA), prevented neuropathic injury–induced phosphorylated serum- and glucocorticoid-inducible kinase 1 (pSGK1)-phosphorylated histone deacetylase (pHDAC4) colocalization. (*A*) Immunohistochemical images showing spinal nerve ligation (SNL) notably enhanced the immunoreactivity of pSGK1 (IV, *green*) and pHDAC4 (V, *red*) compared with the sham group (Sham, I and II, respectively) in the ipsilateral dorsal horn of spinal slices obtained at day 7 after operation (7D). Overlay images and the count of double-labeled neurons show SNL enhanced the colocalized immunoreactivity of pSGK1 with pHDAC4 in the dorsal horn (VI and VIa, *yellow*), which were remarkably reduced by the administration with GSK-650394 (an SGK1 inhibitor; 30 nM, 10 μl; SNL7D + GSK, IX and IXa), but not 14-3-3β messenger RNA-targeting small interfering RNA (SNL 7D + 14-3-3β RNAi, XII and XIIa). (*B*) Statistical analysis showing SNL notably enhanced the count of pSGK1-positive, pHDAC4-positive, and double-labeled dorsal horn that were remarkably reduced by the administration with GSK-650394 (SNL7D + GSK), but not 14-3-3β mRNA-targeting siRNA (SNL 7D + 14-3-3β RNAi, ** $P < 0.01$ compared with the Sham 7D, ##$P < 0.01$ compared with the SNL 7D, n = 7).

In this study, we showed that neuropathic injury provoked spinal SGK1-dependent HDAC4 phosphorylation and cytoplasmic retention. Nevertheless, the precise site where the SGK1-dependent HDAC4 phosphorylation occurs cannot be easily clarified, for which we analyzed the amount of pHDAC4 in dorsal horn homogenate. On the basis of the evidences demonstrated, HDAC4 phosphorylation promotes nuclear export but prevents nuclear import of HDAC4.[24] Moreover, 14-3-3β functions as a cytoplasmic anchoring protein for HDAC4.[25,26] We speculated that HDAC4 phosphorylation possibly occurs in the nucleus and the following association with 14-3-3β restricts HDAC4 location by anchoring this complex in the cytoplasm. Our speculation was derived from findings that the knockdown of 14-3-3β expression exhibited no effect on HDAC4 phosphorylation. However, it did attenuate cytoplasmic HDAC4 accumulation because there is no 14-3-3β available to anchor the HDAC4 shuttled from the nucleus after phosphorylation. Contrarily, if HDAC4 phosphorylation occurs within the cytoplasm, knockdown of 14-3-3β would not affect the amount of cytoplasmic HDAC4 because the degree of HDAC4 phosphorylation kept relatively constant. Our theory was supported by a study, in which either one or combinations of three HDAC4 shuttling–related residues were mutated, demonstrated HDAC4 is predominantly cytoplasmic in wild-type and single mutant cells, whereas it is nuclear in most triple mutant cells.[27] Moreover, results obtained from cultured corticotrophinoma cells provides an indirect evidence by showing that most SGK1 was cytosolic before stimulation, whereas the amount of phosphorylated and nucleus-bound SGK1 increased after dexamethasone activation.[28]

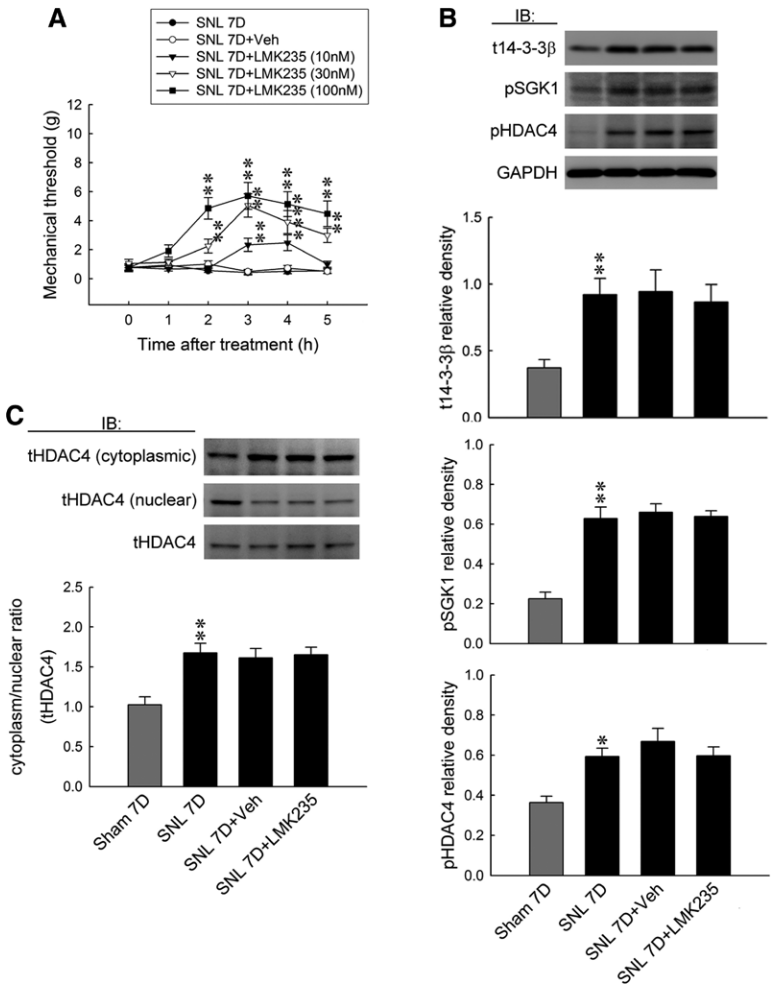

Fig. 8. LMK235 prevented allodynia but not serum- and glucocorticoid-inducible kinase 1 (SGK1) phosphorylation, histone deacetylase (HDAC4) phosphorylation/translocation, and 14-3-3β expression. (*A*) At day 7 (7D) after spinal nerve ligation (SNL), administration with LMK235 (an HDAC4 and 5 inhibitor) increased the withdrawal threshold of the ipsilateral hind paw at hours 2, 3, 4, and 5 after treatment (***P* < 0.01 compared with hour 0, n = 7). (*B* and *C*) Representative Western blot and statistical analysis showing LKM235 exhibited no effect on the SNL-enhanced amount of total 14-3-3β (t14-3-3β), phosphorylated SGK1 (pSGK1), phosphorylated HDAC4 (pHDAC4), and the cytoplasmic/nuclear ratio of total HDAC4 (**P* < 0.05, ***P* < 0.01 compared with the sham operation [Sham] 7D, n = 6). GADPH = glyceraldehyde 3-phosphate dehydrogenase; IB = immunoblotting; Veh = vehicle solution.

We, therefore, propose that neuropathic injury provokes phosphorylation and subsequently nuclear accumulation of SGK1, which is followed by HDAC4 phosphorylation in the nucleus of dorsal horn neurons. However, the precise site where HDAC4 phosphorylation occurs requires further study to be clarified.

Similar to the study of Laffray *et al.*,[10] in this study, we demonstrated that neuropathic injury enhanced the abundance of dorsal horn 14-3-3, a ubiquitous phosphor-binding protein.[29] Conversely, trim-down of spinal 14-3-3β expression ameliorated the developed allodynia. Moreover, accompanied with allodynia, SNL provoked HDAC4 phosphorylation–dependent HDAC4-14-3-3β coupling that restricted location of HDAC4 to the cytoplasm. Although it failed to affect HDAC4 phosphorylation, knockdown of spinal 14-3-3β expression uncoupled HDAC4 from 14-3-3, attenuated cytoplasmic HDAC4 retention, and ameliorated the developed allodynia. In line with evidences showing phosphorylation-dependent HDAC4 coupling with 14-3-3β induces cytosolic HDAC4 accumulation[7] regulates memory-associated synaptic plasticity.[30–32] Our results imply that HDAC4-14-3-3β coupling-dependent cytoplasmic HDAC4 retention is crucial for the neuropathic development, and this machinery is possibly involved in activity-dependent plasticity in general.

To understand the machinery underlying the sensitization of nociception may eventually provide insight into developing medical strategies for pain relief. In this study, we identified spinal HDAC4 functions as a key element important for the neuropathic injury–associated allodynia. Although the use of HDACs-targeting siRNAs/antagonists for therapy garners lots of interest from researchers, we focally knocked-down 14-3-3β instead of HDAC4 expression. Our choice is based on that HDAC4 is abundantly

expressed in the central nervous system of mammalian,[33] and selective HDAC4 knockout, even restricted to the central nervous system, could abolish its inhibition of gene transcription required for normal neural activity, despite it might ameliorate pain hypersensitivity. Our speculation is supported by mounting evidence linking misregulated HDAC activity to degenerative disease models such as Huntington disease,[34] Parkinson disease,[35] Alzheimer disease,[36] motor neuron disorders,[37] and Rubinstein–Taybi syndrome.[38] In contrast, our results showed trim-down of spinal 14-3-3β exhibited almost no effect on the neuropathic injury–induced HDAC4 phosphorylation, implying this protocol has minor impact on HDAC4 activity. We, therefore, suggest that designing 14-3-3β-targeting siRNAs/drugs would be a good starting point for developing medical treatments for neuropathic pain. However, possible side effects of candidate agents need to be verified carefully.

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Competing Interests

The authors declare no competing interests.

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Address correspondence to Dr. Peng: Department of Medicine, Mackay Medical College, No. 46, Sec. 3, Zhongzheng Road, Sanzhi District, New Taipei, Taiwan 25245, Republic of China. hsien.yu@gmail.com. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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