Growth Arrest and DNA-damage–inducible Protein 45β-mediated DNA Demethylation of *Voltage-dependent T-type Calcium Channel 3.2 Subunit* Enhances Neuropathic Allodynia after Nerve Injury in Rats

Cheng-Yuan Lai, B.S., Ming-Chun Hsieh, M.Sc., Yu-Cheng Ho, Ph.D., An-Sheng Lee, Ph.D., Hsueh-Hsiao Wang, Ph.D., Jen-Kun Cheng, M.D., Ph.D., Yat-Pang Chau, Ph.D., Hsien-Yu Peng, Ph.D.

ABSTRACT

Background: Growth arrest and DNA-damage–inducible protein 45β reactivates methylation-silenced neural plasticity-associated genes through DNA demethylation. However, growth arrest and DNA-damage–inducible protein 45β–dependent demethylation contributes to neuropathic allodynia-associated spinal plasticity remains unclear.

Methods: Adult male Sprague–Dawley rats (654 out of 659) received a spinal nerve ligation or a sham operation with or without intrathecal application of one of the following: *growth arrest and DNA-damage–inducible protein 45*β *messenger RNA*–targeted small interfering RNA, lentiviral vector expressing *growth arrest and DNA-damage–inducible protein 45*β, Ro 25–6981 (an NR2B-bearing *N*-methyl-D-aspartate receptor antagonist), or KN-93 (a calmodulin-dependent protein kinase II antagonist) were used for behavioral measurements, Western blotting, immunofluorescence, dot blots, detection of unmodified cytosine enrichment at cytosine-phosphate-guanine site, chromatin immunoprecipitation quantitative polymerase chain reaction analysis, and slice recordings.

Results: Nerve ligation-enhanced growth arrest and DNA-damage–inducible protein 45β expression (n = 6) in ipsilateral dorsal horn neurons accompanied with behavioral allodynia (n = 7). Focal knockdown of growth arrest and DNA-damage–inducible protein 45β expression attenuated ligation-induced allodynia (n = 7) by reducing the binding of growth arrest and DNA-damage–inducible protein 45β to the *voltage-dependent T-type calcium channel 3.2 subunit* promoter (n = 6) that decreased expression of and current mediated by the *voltage-dependent T-type calcium channel 3.2 subunit* (both n = 6). In addition, NR2B-bearing *N*-methyl-D-aspartate receptors and calmodulin-dependent protein kinase II act in an upstream cascade to increase growth arrest and DNA-damage–inducible protein 45β expression, hence enhancing demethylation at the *voltage-dependent T-type calcium channel 3.2 subunit* promoter and up-regulating *voltage-dependent T-type calcium channel 3.2 subunit* expression. Intrathecal administration of Ro 25–6981, KN-93, or a growth arrest and DNA-damage–inducible protein 45β–targeting small interfering RNA (n = 6) reversed the ligation-induced enrichment of unmodified cytosine at the *voltage-dependent T-type calcium channel 3.2 subunit* promoter by increasing the associated 5-formylcytosine and 5-carboxylcytosine levels.

Conclusions: By converting 5-formylcytosine or 5-carboxylcytosine to unmodified cytosine, the NR2B-bearing *N*-methyl-D-aspartate receptor, calmodulin-dependent protein kinase II, or growth arrest and DNA-damage–inducible protein 45β pathway facilitates *voltage-dependent T-type calcium channel 3.2 subunit* gene demethylation to mediate neuropathic allodynia.
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DNA methylation is an epigenetic modification essential for gene silencing and genome stability. Growth arrest and DNA-damage–inducible protein 45 (Gadd45) is known to relieve methylation-induced gene silencing by promoting DNA demethylation, which erases or changes the DNA methylation state of cytosine-phosphate-guanine (CpG) dinucleotides at genomic loci.[1] Among the Gadd45 family members,[2] a growing body of evidence has implicated Gadd45β as a crucial mediator of activity-induced gene-specific demethylation in the central nervous system (CNS).[3] Intriguingly, Gadd45β is known to be required for specifically promoting DNA demethylation or hypomethylation[3,4] and the accompanying transcriptional activation[1,5] of target genes that are essential for forms of spinal plasticity-dependent neuropathic pain.[6,7] Hypomethylated CpG sites

What We Already Know about This Topic

- The methylation of DNA cytosine residues reduces the expression of nearby genes
- Growth arrest and DNA-damage–inducible protein 45β promotes the demethylation of DNA and the expression of pain-related genes

What This Article Tells Us That Is New

- The ligation of nerves in the rat hind limb both caused nociceptive sensitization and expression of growth arrest and DNA-damage–inducible protein 45β (Gadd45β) in spinal cord tissue
- The abundance of Gadd45β controlled the expression of the calcium ion channel *voltage-dependent T-type calcium channel 3.2 subunit* through demethylation, which in turn appeared to modulate nociceptive sensitization

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in the *voltage-dependent T-type calcium channel 3.2 subunit* ($Ca_V3.2$) gene are essential for $Ca_V3.2$ transcription.[8] $Ca_V3.2$ in lamina II and III of the dorsal horn are molecular substrates of neuropathic allodynia.[9] Therefore, the activation of Gadd45β-mediated DNA demethylation, which is required to reactivate the methylation-silenced $Ca_V3.2$ gene, likely represents one of the mechanisms underlying the modulation of spinal plasticity-dependent neuropathic allodynia.

A landmark study showed the application of an *N*-methyl-D-aspartate receptor (NMDAR) agonist activates calmodulin-dependent protein kinase (CaMK), which up-regulates Gadd45β expression, enhances Gadd45β-mediated DNA demethylation, and subsequently induces neural plasticity-related gene transcription in the mammalian brain.[5] Conversely, transfection of cultured neurons with Gadd45β small interfering RNA (siRNA) abolishes NMDAR agonist–induced DNA demethylation of the target gene.[10] The NMDAR agonist–dependent up-regulation of Gadd45 expression in the rat retina was blocked by a calmodulin-dependent protein kinase II (CaMKII)–specific inhibitor.[11] In addition, several *in vivo* studies showed that impaired spinal NR2B-containing NMDAR or CaMKII signaling attenuates nociceptive responses,[12,13] and our previous study demonstrated that experimental neuropathic injury enhances NR2B-containing NMDAR phosphorylation in the dorsal horn.[14] This evidence suggests that Gadd45β-dependent DNA demethylation of target genes underlies NR2B or CaMKII signaling–mediated neural activity or plasticity in the development of neuropathic allodynia. Furthermore, recent work demonstrated that $Ca_V3.2$ is associated with NMDAR-mediated transmission at rat central synapses[15] and regulated by CaMKII to enhance neuronal excitability.[16] Moreover, Ca^{2+}-dependent CaMKII signaling regulates the transcription of Ca^{2+} channels.[17] These observations prompted us to hypothesize that the activation of CaMKII by spinal NR2B-NMDAR phosphorylation contributes to the development of neuropathic allodynia through Gadd45β-mediated $Ca_V3.2$ gene demethylation.

DNA in most mammalian cells displays relatively stable methylation patterns, with 70 to 80% methylated CpG dinucleotides.[18] The active demethylation pathway, which initiates gene transcription, involves the hydroxylation of the methyl group (5-hmC) of methylated cytosine (5-mC), the further oxidation of 5-hmC into 5-formylcytosine and/or 5-carboxylcytosine, and the restoration of unmodified 5-cytosine *via* nucleotide-excision repair.[19–21] Intriguingly, spinal 5-hmC significantly increases in a model of formalin-induced acute inflammatory pain.[22] Moreover, recent data suggest Gadd45β facilitates active DNA demethylation by catalyzing the substitution of 5-mC with unmodified cytosine.[23] Furthermore, Gadd45 has been shown to excise intermediates of 5-mC demethylation, such as 5-formylcytosine and 5-carboxylcytosine, resulting in reversion to unmodified cytosine to complete the active demethylation process.[24] Therefore, we hypothesized that the role of spinal Gadd45β in demethylating the $Ca_V3.2$ promoter through the removal of 5-formylcytosine and/or 5-carboxylcytosine in favor of unmodified cytosine underlies NR2B-containing NMDAR or CaMKII signaling in spinal nerve ligation (SNL)–induced neuropathic allodynia.

Materials and Methods

Animal Preparations

All animal procedures in this study were conducted in accordance with the guidelines of the International Association for the Study of Pain (Washington, DC)[25] and were reviewed and approved by the Institutional Review Board of Taipei Medical University, Taipei, Taiwan. Adult male Sprague–Dawley rats weighing 200 to 250 g were housed at room temperature (23 + 1°C) with a 12-h light–dark cycle (lights on 8:00 AM to 8:00 PM) and were fed food and water *ad libitum*. Animals were randomly allocated to treatment groups using a Research Randomizer (a randomizer on the Web site https://www.randomizer.org/) and the sample size of each group was based on our previous experience. In each group, there were seven rats used for behavioral test and immunohistochemistry, five rats for quantitative reverse-transcription polymerase chain reaction (PCR), and six rats for the Western blot, chromatin immunoprecipitation (ChIP) quantitative PCR, electrophysiologic analysis, dot blot analysis, and measurement of 5-cytosine levels at CpG sites on the $Ca_V3.2$ promoter; the investigators were blinded to the treatment groups for all experiments.

Spinal Nerve Ligation

Spinal nerve ligation rat model was performed as described by previous studies.[26] In brief, rats were deeply anesthetized with isoflurane (5% induction and 2% maintenance in air) and were placed in a prone position. After making an incision, the left L5–L6 spinal nerves were carefully dissected and tightly ligated with 6-0 silk sutures 2 to 5 mm distal to the dorsal root ganglia. After ligation, the incision on the animal was closed. In the sham-operated group, the surgical procedures were identical to the nerve ligation group, except the silk sutures were left unligated.

Intrathecal Catheter

Implantation of intrathecal cannulae was performed as described in our previous study.[27,28] Under anesthesia with isoflurane (5% induction and 2% maintenance in air),

C.-Y.L. and M.-C.H. contributed equally to this study.

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PE-10 silastic tubing was implanted in the lumbar enlargement of the spinal cord. The outer part of the catheter was plugged and immobilized onto the skin on closure of the wound. Five rats that displayed neurologic deficits after surgery were euthanized and excluded from statistical analyses.

Behavioral Studies

Tactile sensitivity was assessed by measuring each rat's paw withdrawal threshold in response to probing with von Frey monofilaments (Stoelting, USA) according to a modification of a previously described method.[29] In brief, rats were placed individually in an opaque plastic cylinder, which was placed on a wire mesh. The animals were habituated for 1 h to allow acclimatization to the test environment before each test. After acclimatization, calibrated von Frey filaments of incremental stiffness (0.07 to 26.0 g) were applied serially to the paw in ascending or descending order of stiffness depending on the foot-withdrawal response of the rat. Each trial of stimuli consisted of five applications of the filament every 4 s to the plantar surface of the left hind paw perpendicularly for approximately 3 to 4 s. Brisk foot withdrawals (at least three out of five times the filament was applied) in response to normally innocuous mechanical stimuli using von Frey filaments were considered positive; a lack of response was considered negative. Depending on the positive or negative response, subsequent filaments were applied in order of descending and ascending intensity, respectively. Motor function was assessed using an accelerating rotarod apparatus (LE8500; Ugo Basile, Italy). For acclimatization, the animals were subjected to three training trials at 3- to 4-h intervals on 2 separate days. During the training sessions, the rod was set to accelerate from 3 to 30 rpm over a 180-s period. During the test session, the performance times of rats were recorded up to a cutoff time of 180 s. Three measurements were obtained at intervals of 5 min and were averaged for each test.

Locomotor Activity

The open field test was conducted to determine whether SNL and the additional treatments affect locomotor activity in rats. Each rat was placed in the center of a black arena (50 cm × 50 cm, 40 cm high). A video camera system was used to record the rats' activity for 30 min. Locomotor activity including the traveling distance (centimeters), rears duration, and number of rears was analyzed using EthoVision XT tracking software (Noldus Information Technology, The Netherlands).

Western Blotting

The dissected dorsal horn (L4–L5) and dorsal root ganglion (DRG; L4–L5) sample were homogenized in 25 mM Tris-hydrochloride, 150 mM NaCl, 1% Tergitol-type 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 rpm, 20 min, 4°C). All the protein concentrations were determined using a bicinchoninic acid assay. The supernatant was separated on an acrylamide gel and transferred to a polyvinylidene difluoride membrane, which was then incubated (1 h, room temperature) in either rabbit anti-Gadd45β (1:1,000; Santa Cruz Biotechnology, USA), rabbit anti-$Ca_V3.2$ (1:1,000; Santa Cruz Biotechnology), rabbit anti–phosphorylated NR2B (pNR2B; 1:1,000; Millipore, USA), rabbit anti–phosphorylated CaMKII (pCaMKII; 1:1,000; Sigma-Aldrich, China), or mouse anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:4,000; Santa Cruz Biotechnology). The blots were washed and incubated (1 h, room temperature) in peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG; 1:8,000; Jackson ImmunoResearch, USA) or goat anti-mouse IgG (1:8,000; Jackson ImmunoResearch). The protein bands were visualized using an enhanced chemiluminescence detection kit (ECL Plus; Millipore) and then subjected to a densitometric analysis with Science Lab 2003 (Fuji, Japan).

Immunofluorescence

After perfusion (100 ml phosphate buffered saline [PBS] followed by 300 ml paraformaldehyde, 4%, in PBS, pH 7.4), the spinal cord samples were harvested (L4–L5), postfixed (4°C for 4 h), and cryoprotected overnight in sucrose solution (30%). The samples were double labeled to investigate the interactions between Gadd45β and neuronal, glial, or microglia markers; specifically, the spinal sections were incubated overnight (4°C) with a mixture of rabbit anti-Gadd45β (1:100; Santa Cruz Biotechnology) and mouse monoclonal antineuronal nuclear antigen (a neuronal marker; 1:500; Millipore), mouse antiglial fibrillary acidic protein (a marker of astroglial cells; 1:1,000; Millipore), or mouse antiintegrin αM (OX-42, a marker of microglia; 1:1,000; Santa Cruz Biotechnology). After three rinses with PBS, the sections were then incubated (1 h, 37°C) with Alexa Fluor 488 (1:1,500; Invitrogen, USA) and Alexa Fluor 594 (1:1,500; Invitrogen). When examining the interaction among Gadd45β, pNR2B, and pCaMKII, the specific antibodies were mixed with ×10 reaction buffer (Mix-n-Stain; Biotium, USA) with the antibody solution at a ratio of 1:10. The solution was then transferred to a vial containing dye (CF; Biotium) and incubated in the dark (30 min, room temperature). The spinal cord sections were sequentially incubated (overnight, 4°C) with diluted solutions—*i.e.*, rabbit anti-Gadd45β (1:100; Santa Cruz Biotechnology), rabbit anti-pNR2B (1:200; Biorbyt, USA), and rabbit anti-pCaMKII (1:100; Santa Cruz Biotechnology)—and were washed five times between each incubation. Spinal sections were subsequently rinsed in PBS, and coverslips were applied. When these fluorescent markers are excited, they can easily be detected using a camera-coupled device (X-plorer; Diagnostic Instruments, Inc., USA) using fluorescence microscopy (LEICA DM2500, Germany). For quantity measurement of immunofluorescent intensity, cell counting in the superficial dorsal horn (lamina II) was

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carried out under a microscope at a magnification of ×200. Five sections from each spinal cord were selected, and seven animals were analyzed in each group. Images were analyzed with ImageJ software (National Institutes of Health, USA).

Dot Blot Analysis

The global levels of 5-formylcytosine and 5-carboxylcytosine were assessed using the dot blot analysis. The dissected dorsal horn (L4–L5) was collected and grounded in liquid nitrogen and stored at −80°C. After genomic DNA extraction from tissues using a DNA extraction kit (Qiagen, Germany), the isolated DNA (100 ng per sample) was denatured in 0.1 M NaOH for 10 min at 95°C and then neutralized with 1 M NH_4OAc on ice. Labeled samples were spotted on a positively charged nitrocellulose membrane, which was air-dried at room temperature. After ultraviolet cross-linking at 120,000 μJ/cm^2 for 30 s, the membrane was blocked with 5% milk at room temperature for 1 h. After washing, the membrane was incubated with the primary antibody, *i.e.*, rabbit anti–5-formylcytosine (1:5,000; ActiveMotif, USA) or rabbit anti–5-carboxylcytosine (1:2,000; ActiveMotif) at 4°C overnight. After incubating with primary antibody, the membrane was washed and incubated in goat anti-rabbit IgG (1:8,000; Jackson ImmunoResearch) for 1 h at room temperature. The blots were visualized using an enhanced chemiluminescence detection kit (ECL Plus; Millipore) and then subjected to densitometric analysis using Science Lab 2003 (Fuji).

Methylene Blue Staining

The dot blot membrane was hybridized with 0.02% methylene blue in 0.3 sodium acetate (pH 5.2) to stain DNA for 10 min. After washing and taking photographs, densitometric analysis of the methylene blue staining was performed with Science Lab 2003 (Fuji) to validate equal DNA loading.

Quantitative Reverse-transcription Polymerase Chain Reaction

In brief, the dissected dorsal horn (L4–L5) and DRG (L4–L5) were quickly removed and completely submerged in a sufficient volume of RNAlater solution (AM7021; Ambion, USA) overnight at 4°C to allow thorough penetration of the tissue and then transferred to 80°C. Total RNA was isolated under ribonuclease-free conditions using RNA isolation kits (74106; Qiagen, USA). Reverse transcription was performed using complementary DNA reverse-transcription kits (205311; Qiagen, USA). Real-time PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, USA). TaqMan Universal PCR Master Mix (2×) and TaqMan gene expression assay probes for target genes were GAPDH (Rn99999916_s1; Applied Biosystems) and $Ca_V3.2$ (Rn.PT.58.44897351; IDT, USA). Reactions (total volume, 20 μl) were performed by incubating at 95°C for 20 s, followed by 40 cycles of 1 s at 95°C and 20 s at 60°C. Relative messenger RNA (mRNA) levels were calculated according to the $2^{-\Delta\Delta CT}$ method.[30] All CT values were normalized to GAPDH.

Measurement of 5-Cytosine Levels at CpG Sites on the $Ca_V3.2$ Promoter

The dissected spinal cord (L4–L5) was collected, grounded in liquid nitrogen, and stored at −80°C. Genomic DNA was extracted using a DNA extraction kit (Qiagen, Germany). Purification of 5-cytosine–enriched DNA fragments was performed using the UnMethylCollector Kit (ActiveMotif). After purification, samples were prepared for quantitative reverse-transcription polymerase chain reaction using Power SYBR Green (Applied Biosystems) on an Applied Biosystems® StepOne machine (Applied Biosystems). Data are presented as percent input, which is calculated as $100 \times 2^{-\Delta Ct}$, where ΔCt is the average Ct value of the triplicate input minus the average Ct value of the triplicate sample. The primers used to amplify the $Ca_V3.2$ promoter were as follows: 5′-GAGAGAGGGCAGGAGGTGAC-3′ and 5′-TGGGACCCTTTGAACTTGAG-3′.

Chromatin Immunoprecipitation Quantitative Polymerase Chain Reaction

Chromatin immunoprecipitation was performed using a ChIP Kit (Millipore) according to a modified protocol from the manufacturer. Dissected spinal cord samples were cut into small pieces (1 to 2 mm^3) using razor blades, treated with fresh 1% paraformaldehyde in PBS buffer, and gently agitated for 10 min at room temperature to cross-link proteins to DNA. Then, the tissue was washed and resuspended in lysis buffer, and the lysates were sheared by sonication to generate chromatin fragments with an average length of 200 to 1,000 bp. One percent of the sonicated chromatin was saved as an input control for quantitative reverse-transcription polymerase chain reaction. The chromatin was then immunoprecipitated for 2 h at room temperature with rabbit anti-Gadd45β (1:1,000; Santa Cruz Biotechnology) or an equivalent amount of control IgG. The protein–DNA immunocomplexes were precipitated overnight using protein G magnetic beads at 4°C. After the beads were washed, they were resuspended in ChIP elution buffer, incubated with proteinase K at 62°C for 2 h, and then incubated at 95°C for 10 min to reverse the protein–DNA cross-links. ChIP signals were quantified by quantitative reverse-transcription polymerase chain reaction analysis with a 7500 Real-Time PCR System (Applied Biosystems). The ChIP primer sequences were as follows: 5′-GAGAGAGGGCAGGAGGTGAC-3′ and 5′-TGGGACCCTTTGAACTTGAG-3′.

Small Interfering RNA

The 19-nucleotide siRNA duplex used for Gadd45β was 5′-GAAGAGAGCAGAGGCAAUA-3′, and the missense nucleotide sequence was 5′-UGAUAUUACCCUGAAUAUG-3′. The missense or siRNA construct was intrathecally administered using a polyethyleneimine (10 μl; Dharmacon, USA)–based gene-delivery system into the dorsal subarachnoid space (L4–L5) of animals through the implanted catheter (daily for 4 days).

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Preparation and Injection of Lentiviral Vectors

A rat lentiviral vector expressing *Gadd45β* (NM_001008321) was purchased from a medical supplier (Applied Biological Materials Inc., Canada). To produce lentiviral particles, transfer vectors encoding Gadd45β-2A-GFP or GFP alone (as a control), pMD2.G, and psPAX2 were cotransfected into 293T cells using a liposome delivery method. After 6 h of incubation, the culture medium was discarded and the cultured cells were washed twice with PBS. Culture medium containing lentiviruses was collected at 48 h after the washing, cleared by centrifugation at 300*g* at 4°C for 10 min and filtered through a 0.22-mm pore size cellulose acetate filter. The lentiviruses were then concentrated by ultracentrifugation at 3,000 rpm at 4°C for 150 min. The titer was determined using a quantitative reverse-transcription polymerase chain reaction Lentivirus Titration Kit (Abm Inc., Canada). The rats received a single injection of 15 μl of lentiviral vector expressing *Gadd45β* (2.4×10^7 U/ml) or control vector (2.2×10^7 U/ml) using an intrathecal catheter removed immediately after vector injection.

Spinal Slice Preparations

Under anesthesia with isoflurane, rats underwent a laminectomy for removal of the lumbar spinal cord. The lumbar spinal cord section was placed in ice-cold dissecting solution: 234 mM sucrose, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 12 mM glucose, and 25 mM $NaHCO_3$ and bubbled with 95% O_2/5% CO_2. After dissection, slices were equilibrated in artificial cerebrospinal fluid at room temperature for at least 1 h before recording. The artificial cerebrospinal fluid consisted of the following: 117 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11.4 mM dextrose bubbled with 95% O_2/5% CO_2, pH 7.4. The external solution for T-type calcium channel current recording experiments contained 140 mM tetraethylammonium-Cl, 2 mM $BaCl_2$, 1 mM $MgCl_2$, 2.5 mM CsCl, 10 mM HEPES, and 10 mM glucose, pH 7.3. To the external solution were also added 10 μM bicuculline methiodide, 50 μM nifedipine, 3 μM ω-conotoxin MVIIC, and 1 μM tetrodotoxin to isolate T-type currents.[31] During recordings, a spinal slice was mounted in a submerged recording chamber and continuously perfused with oxygenated external solution at 3 to 4 ml/min.

Whole Cell Patch Clamp Recordings

The lamina II of the spinal cord, which primarily gives rise to the nociceptive unmyelinated fiber,[32] was identified on an upright fixed-stage IR-DIC microscope (BX51WI; Olympus, Japan). The neuron in the outer region of lamina II, which was identified as the region 100 to 130 μm from the dorsal edge of the white matter, was selected for whole cell patch clamp recordings as previously described.[33,34] Glass pipettes (5 to 8 MΩ resistance) were filled with an internal solution containing 110 mM Cs^+ gluconate, 5 mM tetraethylammonium, 5 mM QX314, 0.5 mM $CaCl_2$, 5 mM BAPTA, 10 mM HEPES, 5 mM MgATP, and 0.33 mM GTP-Tris, pH 7.3, 280 mOsm/l. Currents were measured in response to 400 ms voltage clamp pulses from a holding potential of −80 mV to test potentials between −80 and +60 mV. Electrophysiologic signals were acquired using an Axon setup (Molecular Devices/Axon Instruments, USA). Signals were sampled by pCLAMP 9.2 *via* an amplifier (Axopatch 200B, Molecular Devices) and an AD converter (Digidata 1322A, Molecular Devices), filtered at 2 to 5 kHz, digitized at 10 kHz, and stored for off-line analysis.

Drug Application

Ro 25–6981 (an NR2B activation antagonist; 100 nM, 10 μl; Tocris Bioscience, USA; intrathecal; dimethyl sulfoxide) and KN-93 (a selective CaMKII antagonist; 50 nM, 10 μl; Tocris Bioscience; intrathecal; dimethyl sulfoxide) were intrathecally administered daily for 4 days from days 3 to 6 after SNL. L-Ascorbic acid (a selective $Ca_V3.2$ antagonist; 10, 30, and 100 μM, 10 μl; Tocris Bioscience; intrathecal; dimethyl sulfoxide) was intrathecally administered by a bolus injection. A vehicle solution of the volume identical to that of the tested agents was dispensed to serve as a control.

Data Analysis

All data in this study were analyzed using SigmaPlot 10.0 (Systat Software, USA) or Prism 6.0 (GraphPad, USA) and were expressed as mean ± SD. A two-way ANOVA was used to assess changes in values for serial measurements over time, and a Tukey *post hoc* test was used to compare the means of groups (fig. 1A). A one-way ANOVA was used to assess changes in values for serial measurements over time, and paired two-tailed Student's *t* tests were used to compare the means of groups (fig. 1B). Statistical comparison was performed by a one-way ANOVA followed by Tukey test for *post hoc* analysis (figs. 1C and 2A). Figure 2B is the same as figure 1A. Figure 2, C and D, are the same as figure 1B. Figure 2, E and F, are the same as figure 1C. Paired two-tailed Student's *t* tests were used to compare the mean between groups in figure 2G. Figure 2H is the same as figure 1B. Figure 3A is no statistical analysis. Figure 3B–D are the same as figure 1C. Figure 4A is the same as figure 1A. Figure 4B–F are the same as figure 1C. Figure 4G is the same as figure 2G. Figure 4, H and I, are the same as figure 1C. Figure 4J is the same as figure 2G. Figure 4K is the same as figure 1C. Figure 5, A and B, are not statistical analysis. Figure 5C is the same as figure 1C. Figures 6A–D, 7A, and 8A–C are the same as figure 1C. Significance was set at $P < 0.05$. No statistical power calculation was conducted before our study, and the sample size selected was based on our previous experience using this design.

Results

Nerve Ligation Enhances Gadd45β Expression in Dorsal Horn Neurons and Provokes Behavioral Allodynia

To characterize the role of spinal Gadd45β in the development of neuropathic allodynia, we first examined the

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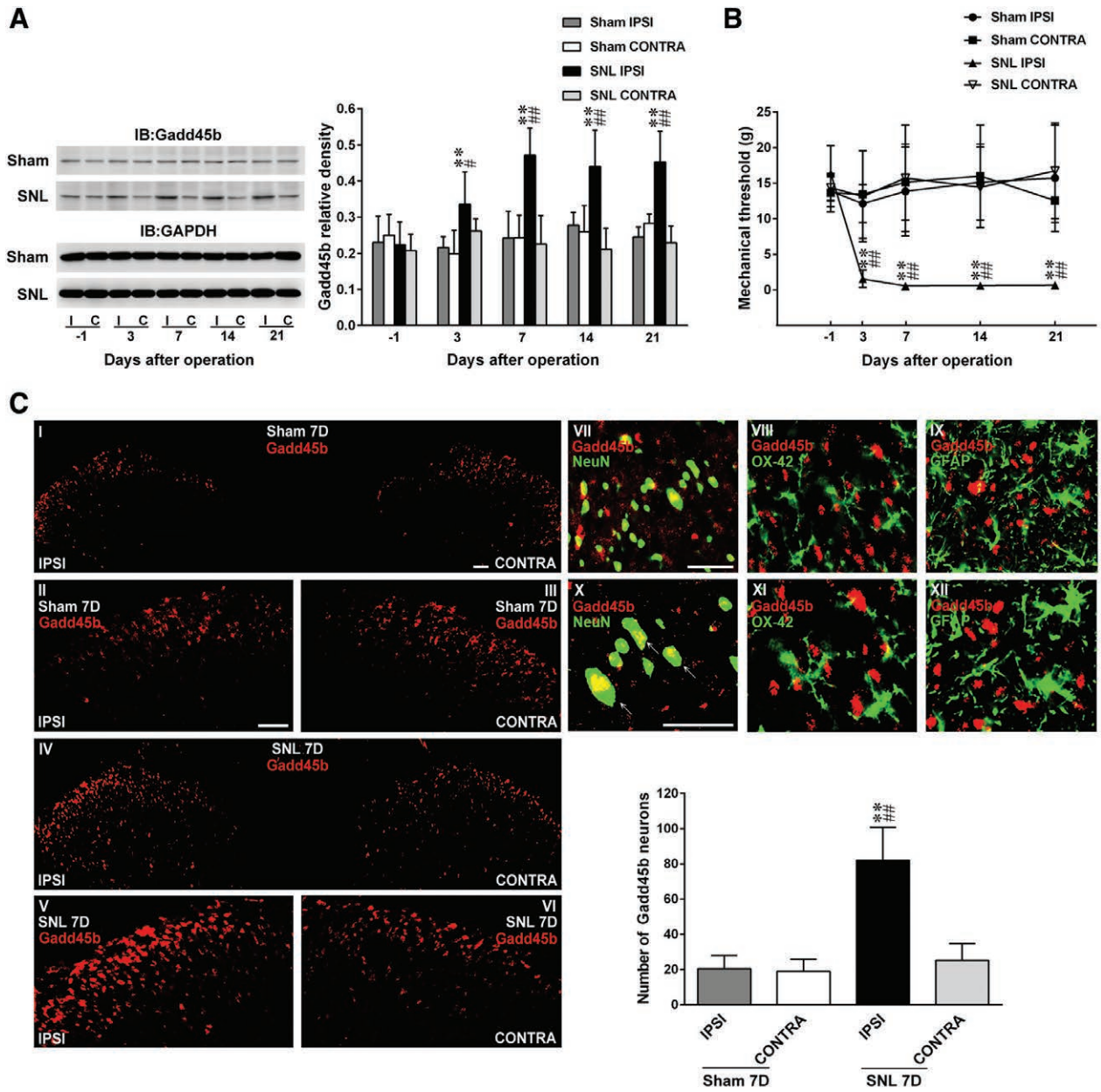

Fig. 1. Spinal nerve ligation (SNL) up-regulates growth arrest and DNA-damage–inducible protein 45β (Gadd45β) expression in spinal dorsal horn neurons, accompanied by behavioral allodynia. (*A*) Representative Western blot and statistical analysis (normalized to glyceraldehyde 3-phosphate dehydrogenase protein [GAPDH]) revealing that whereas sham operation (Sham) exhibited no effect, SNL increased the abundance of Gadd45β on days 3, 7, 14, and 21 postoperation specifically in the ipsilateral (I and IPSI), but not the contralateral (C and CONTRA), dorsal horn. ***P* < 0.01 *versus* Sham IPSI. #*P* < 0.05. ##*P* < 0.01 *versus* SNL day −1. n = 6. (*B*) The withdrawal threshold of the ipsilateral (SNL IPSI), but not the contralateral (SNL CONTRA), hind paw decreased on days 3, 7, 14, and 21 after SNL (von Frey test). ***P* < 0.01 *versus* Sham IPSI. ##*P* < 0.01 *versus* SNL day −1. n = 7. (*C*) In the ipsilateral dorsal horn (IPSI), SNL (SNL 7D) increased the immunofluorescence of Gadd45β (*red*; IV and V), which colocalized (*yellow*) with neuronal (NeuN, a marker of neurons; *green*; VII and X) but not microglial (OX-42, a marker of microglia; *green*; VIII and XI) or astrocyte (glial fibrillary acidic protein, a marker of astrocytes; *green*; IX and XII) markers, on day 7 postoperation. *Scale bar* = 50 μm. *Thickness* = 50 μm. ***P* < 0.01 *versus* Sham IPSI. ##*P* < 0.01 *versus* SNL CONTRA. n = 7. Data represent mean ± SD. IB = immunoblotting.

abundance of Gadd45β in the dorsal horn in response to experimental nerve injury. Western blotting revealed that SNL significantly increased the amount of Gadd45β in the ipsilateral, but not contralateral, dorsal horn on days 3, 7, 14, and 21 after the operation (fig. 1A; 0.22 ± 0.06, 0.33 ± 0.08, 0.47 ± 0.10, 0.43 ± 0.10, and 0.45 ± 0.08; n = 6). Moreover, the enhanced Gadd45β expression was temporally aligned with SNL-induced tactile allodynia, as shown by significant

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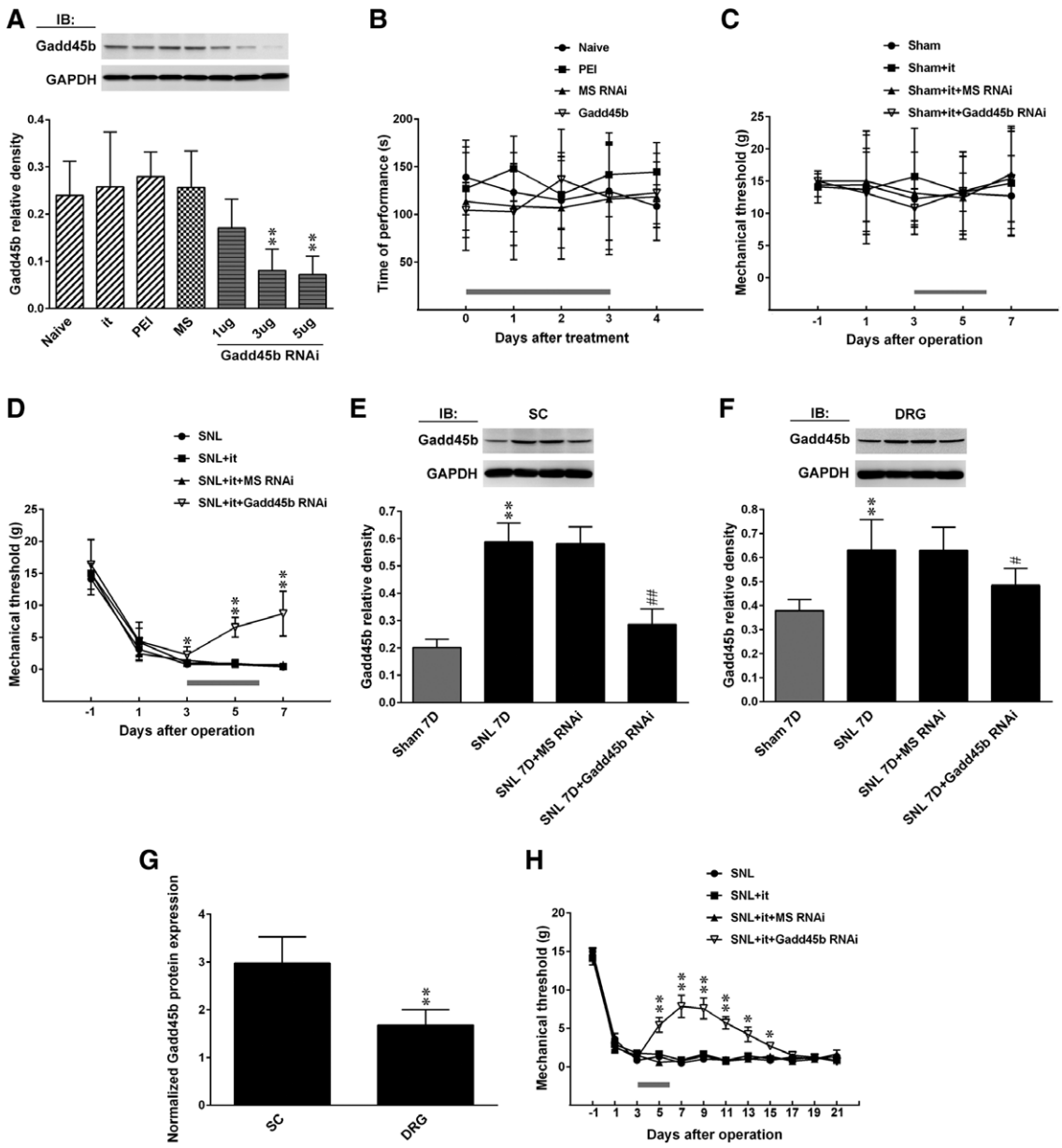

Fig. 2. Focal knockdown of spinal growth arrest and DNA-damage–inducible protein 45β (Gadd45β) expression relieves spinal nerve ligation (SNL)–induced allodynia. (*A*) Representative Western blot and statistical analysis (normalized to glyceraldehyde 3-phosphate dehydrogenase protein [GAPDH]) demonstrating that spinal Gadd45β mRNA-targeting siRNA (Gadd45β RNAi; 1, 3, and 5 μg; 10 μl; once daily for 4 days), but not missense siRNA (MS RNAi; 5 μg, 10 μl), polyethylenimine (PEI, a transfection reagent; 10 μl), or implantation of the intrathecal catheter alone (it), led to a dose-dependent decrease in Gadd45β levels in the ipsilateral dorsal horn of naive rats (Naive). $^{**}P < 0.01$ *versus* Naive. n = 6. (*B*) The application of Gadd45β mRNA–targeting siRNA (Gadd45β RNAi; 5 μg, 10 μl; once daily for 4 days) failed to produce motor deficits in rats (rotarod test). The *gray bar* at the bottom indicates the duration of intrathecal administration. n = 7. (*C*, *D*) Although no significant effect on paw withdrawal threshold was observed in sham-operated animals (Sham), focal knockdown of spinal Gadd45β expression (SNL + it + Gadd45β RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL) ameliorated the SNL-decreased paw withdrawal threshold on days 5 and 7 postoperation (von Frey test). The *gray bar* at the bottom indicates the duration of spinal administration. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* SNL. n = 7. (*E*, *F*) Representative Western blot and statistical analyses (normalized to GAPDH) showing that, on day 7 postoperation (7D), the SNL-enhanced Gadd45β expression in the ipsilateral dorsal horn of the spinal cord (SC) and dorsal root ganglion (DRG) were attenuated by Gadd45β mRNA–targeting siRNA (SNL 7D + Gadd45β RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL). $^{*}P < 0.01$ *versus* Sham 7D. #$P < 0.05$. ##$P < 0.01$ *versus* SNL 7D. n = 6. (*G*) The SNL-enhanced Gadd45β expression in the DRG was markedly lesser than that in the dorsal horn (SC; normalized to Sham 7D). $^{*}P < 0.01$ *versus* SC. n = 6. (*H*) Treatment of Gadd45β mRNA–targeting siRNA (SNL + it + Gadd45β RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL) ameliorated the SNL-induced mechanical allodynia from days 5 to 15 after operation. $^{*}P < 0.05$. $^{**}P < 0.01$ *versus* SNL. n = 7. Data represent mean ± SD. IB = immunoblotting.

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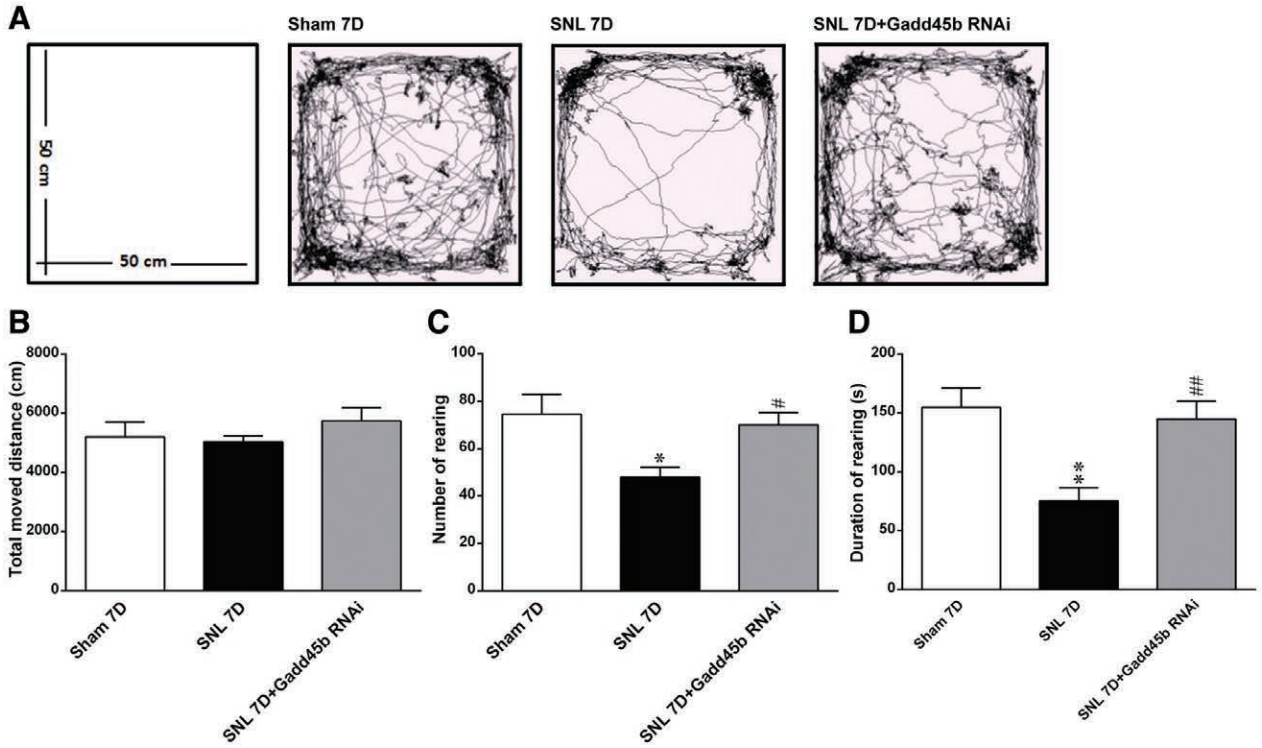

Fig. 3. Focal knockdown of spinal growth arrest and DNA-damage–inducible protein 45β (Gadd45β) expression ameliorates nonevoked pain-like behaviors. (*A*) Schematic diagram of the open-field arena and representative tracks of rat movement in this arena during 30 min. (*B–D*) Averaged bar charts of total moved distance, number of rearing, and duration of rearing obtained on day 7 postoperation from animals received sham operation (Sham 7D) and spinal nerve ligation (SNL 7D) without or with administration with Gadd45β mRNA–targeted siRNA (SNL 7D + Gadd45β RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL). **P* < 0.05. ***P* < 0.01 *versus* Sham 7D. #*P* < 0.05. ##*P* < 0.01 *versus* SNL 7D. n = 6 to 9. Data represent mean ± SD.

decreases in the withdrawal threshold of the ipsilateral hind paw at the same time points (fig. 1B; 16.37 ± 3.88, 1.55 ± 1.22, 0.56 ± 0.54, 0.60 ± 0.46, and 0.66 ± 0.54; n = 7). To visualize the cellular location of SNL-induced enhanced Gadd45β expression, spinal slices were labeled with specific antibodies on day 7 after the operation, the time point at which the behavioral hypersensitivity reached steady state and maximal spinal Gadd45β expression was observed. As anticipated, SNL increased the immunofluorescence of Gadd45β in the ipsilateral dorsal horn (Sham IPSI, 20 ± 7; Sham contralateral [CONTRA], 19 ± 6; SNL IPSI, 82 ± 18; SNL CONTRA, 25 ± 9; n = 7), and double labeling revealed that Gadd45β immunofluorescence colocalized with neuronal, but not microglial or astrocyte, markers (fig. 1C). These results suggest that neuropathic injury induces nociceptive hypersensitivity associated with enhanced Gadd45β expression selectively in ipsilateral dorsal horn neurons.

Knockdown of Spinal Gadd45β Expression Ameliorates SNL-induced Allodynia

To provide further evidence supporting the role of spinal Gadd45β in the development of neuropathic allodynia, we generated rats in which spinal Gadd45β expression was focally knocked down through daily intrathecal administration of an antisense siRNA specifically targeting Gadd45β mRNA. First, Western blotting demonstrated a dose-dependent decrease in the abundance of Gadd45β in dorsal horn samples from naive rats after intrathecal injection of a Gadd45β mRNA–targeted siRNA (fig. 2A; 1 μg, 0.17 ± 0.06; 3 μg, 0.08 ± 0.04; 5 μg, 0.07 ± 0.03; 10 μl each; once daily for 4 days; n = 6) but not with injection of a missense siRNA (5 μg, 10 μl), polyethylenimine (a transfection reagent, 10 μl), or intrathecal catheter implantation alone, indicating that spinal Gadd45β expression was specifically knocked down by the targeted siRNA. Rotarod analysis showed no significant difference in motor performance among the naive and polyethylenimine (10 μl)–treated, missense siRNA (5 μg, 10 μl)–treated, or Gadd45β mRNA–targeted siRNA (5 μg, 10 μl)–treated groups (fig. 2B), suggesting that neither the control procedures nor spinal Gadd45β knockdown led to motor deficits in rats. The results of the von Frey test showed that while there was no effect on the withdrawal threshold of sham-operated animals (fig. 2C), daily administration of a Gadd45β mRNA–targeted siRNA (5 μg, 10 μl; once daily from days 3 to 6 postoperation) partially ameliorated SNL-induced behavioral allodynia, as evidenced by a significant increase in the withdrawal threshold on days 5 and 7 after the operation (fig. 2D; 6.57 ± 1.51 and 8.71 ± 3.49; n = 7). Moreover, a Gadd45β mRNA–targeted siRNA (5 μg, 10 μl) significantly decreased SNL-enhanced Gadd45β expression

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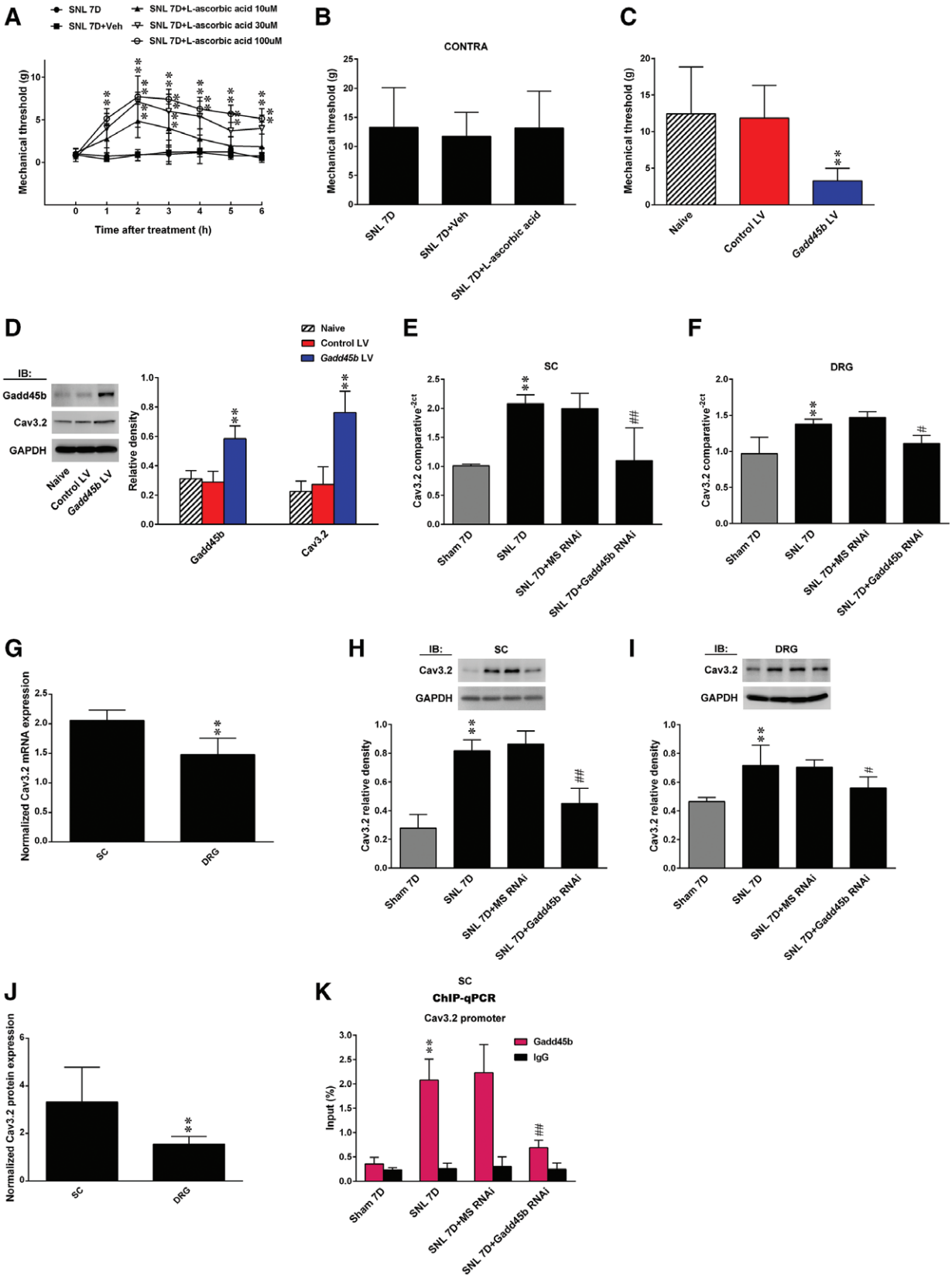

Fig. 4. Spinal nerve ligation (SNL) increases growth arrest and DNA-damage–inducible protein 45β (Gadd45β) binding to the *voltage-dependent T-type calcium channel 3.2 subunit* ($Ca_V3.2$) gene promoter to enhance $Ca_V3.2$ expression in the dorsal horn. (*A*, *B*) Intrathecal administration with L-ascorbic acid (a selective $Ca_V3.2$ antagonist; day 7 after spinal nerve ligation

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Fig. 4. (*Continued*). [SNL 7D] + L-ascorbic acid; 10, 30, and 100 μM, 10 μl), but not the vehicle solution (SNL 7D + Veh, 10 μl), increased the withdrawal threshold of the ipsilateral hind paw on SNL 7D. Nevertheless, both these treatments failed to affect the contralateral hind paw at 2 h after injection (SNL 7D + L-ascorbic acid; 100 μM, 10 μl; von Frey test). ***P* < 0.01 *versus* SNL 7D. n = 7. (*C*) Intrathecal injections of lentiviral vector expressing Gadd45β into naive rat (Gadd45β LV; 2.4×10^7 U/ml, 15 μl) resulted in a decreased withdrawal threshold on day 14 after treatment. Intrathecal injections of control vector into naive rat (Control LV; 2.2×10^7 U/ml, 15 μl). ***P* < 0.01 *versus* Naive. n = 7. (*D*) Representative Western blot and statistical analyses (normalized to glyceraldehyde 3-phosphate dehydrogenase protein [GAPDH]) showing that, on day 14 after treatment, intrathecal injections of lentiviral vector expressing Gadd45β (Gadd45β LV; 2.4×10^7 U/ml, 15 μl), not control vector (Control LV; 2.2×10^7 U/ml, 15 μl), into naive rat (Gadd45β LV; 2.4×10^7 U/ml, 15 μl) increased Gadd45β and $Ca_V3.2$ protein levels in ipsilateral dorsal horn. ***P* < 0.01 *versus* Naive. n = 6. (*E–J*) Representative reverse-transcription polymerase chain reaction, Western blot, and statistical analysis (normalized to GAPDH) revealing that, compared with sham operation (Sham 7D), spinal nerve ligation (SNL 7D) increased $Ca_V3.2$ transcription or expression in ipsilateral dorsal horn of the spinal cord (SC) and dorsal root ganglion (DRG) samples harvested on day 7 postoperation, which was reversed by intrathecal application of Gadd45β mRNA–specific antisense siRNA (SNL 7D + Gadd45β RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL). Missense siRNA (MS RNAi; 5 μg, 10 μl). ***P* <0.01 *versus* Sham 7D. #*P* < 0.05. ##*P* < 0.01 *versus* SNL 7D. n = 6. The SNL-enhanced $Ca_V3.2$ transcription or expression in the DRG was markedly lesser than that in the dorsal horn (SC; normalized to Sham 7D). ***P* < 0.01 *versus* SC. n = 6. (*K*) Chromatin immunoprecipitation (ChIP) quantitative reverse-transcription polymerase chain reaction analysis showed that intrathecal application of Gadd45β mRNA–specific antisense siRNA (SNL 7D + Gadd45β RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL) reversed the SNL-enhanced binding of Gadd45β to the $Ca_V3.2$ promoter region in the ipsilateral dorsal horn on day 7 postoperation. ***P* < 0.01 *versus* Sham 7D. ##*P* < 0.01 *versus* SNL 7D. n = 6. Data represent mean ± SD. IB = immunoblotting.

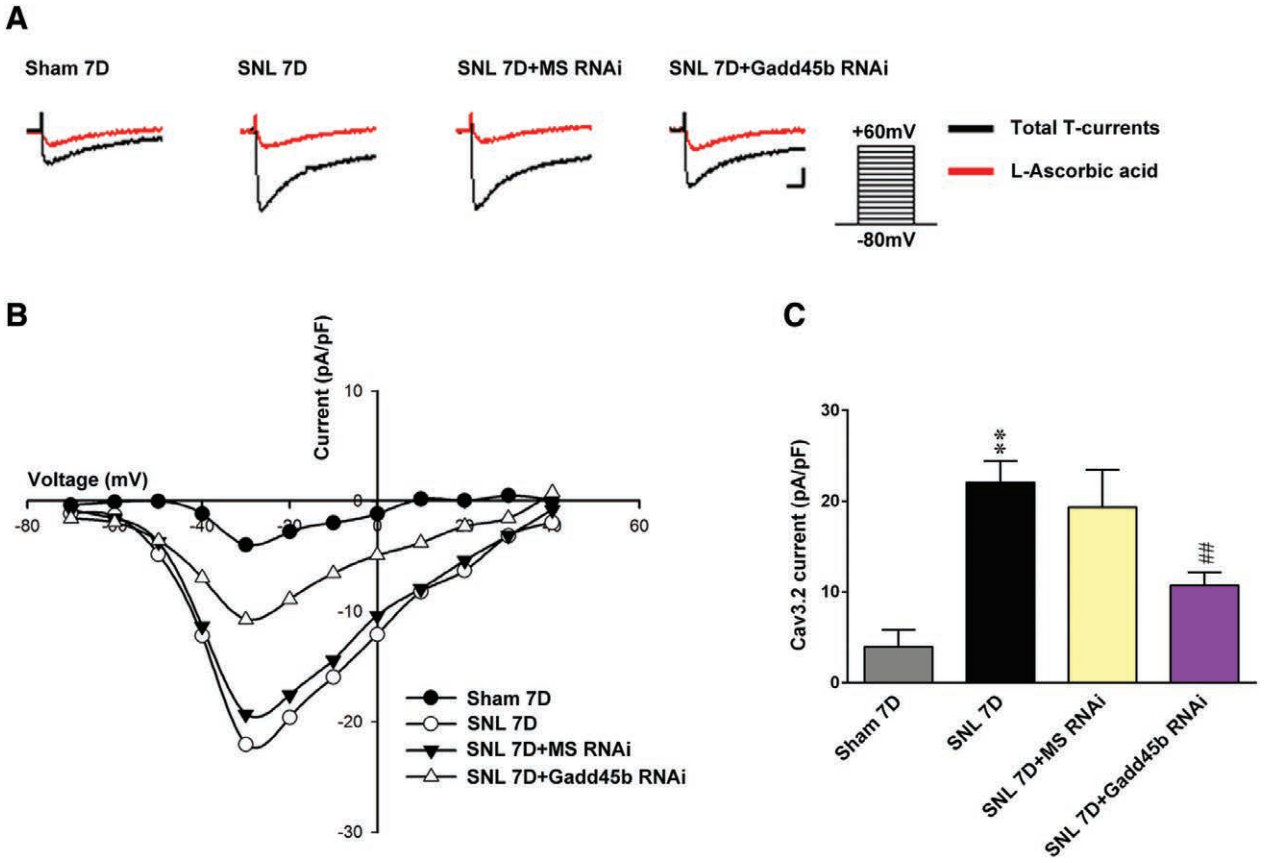

Fig. 5. Knockdown of growth arrest and DNA-damage–inducible protein 45β (Gadd45β) expression restores nerve ligation-induced enhanced *voltage-dependent T-type calcium channel 3.2 subunit* ($Ca_V3.2$) currents in spinal dorsal horn neurons. (*A*) Representative traces of total voltage-dependent T-type currents (Total T-type currents) and $Ca_V3.2$ currents in spinal dorsal horn neurons on day 7 postoperation dissected from sham-operated (Sham 7D) and spinal nerve ligation (SNL 7D) rats as well as SNL rats treated with missense siRNA (SNL 7D + MS RNAi; 5 μg, 10 μl) and Gadd45β-targeting siRNA (SNL 7D + Gadd45β RNAi; 5 μg, 10 μl). L-Ascorbic acid, a $Ca_V3.2$ antagonist (300 μM). (*B*) The current–voltage (I–V) curves of $Ca_V3.2$-dependent currents in spinal dorsal horn neurons from sham (Sham 7D) and SNL rats (SNL 7D) and SNL rats treated with Gadd45β-targeting siRNA (SNL 7D + Gadd45β RNAi) or missense siRNA (SNL 7D + MS RNAi). When compared with the sham operation, SNL increases peak currents of the I–V curve that is reversed by that intrathecally daily application of Gadd45β-targeting siRNA but not the missense siRNA. (*C*) Peak values of the $Ca_V3.2$-dependent current densities (pA–pF) showing reduced the peak amplitude of $Ca_V3.2$ currents compared with SNL group. ***P* < 0.01 *versus* Sham 7D. ##*P* < 0.01 *versus* SNL 7D. n = 6. Data represent mean ± SD.

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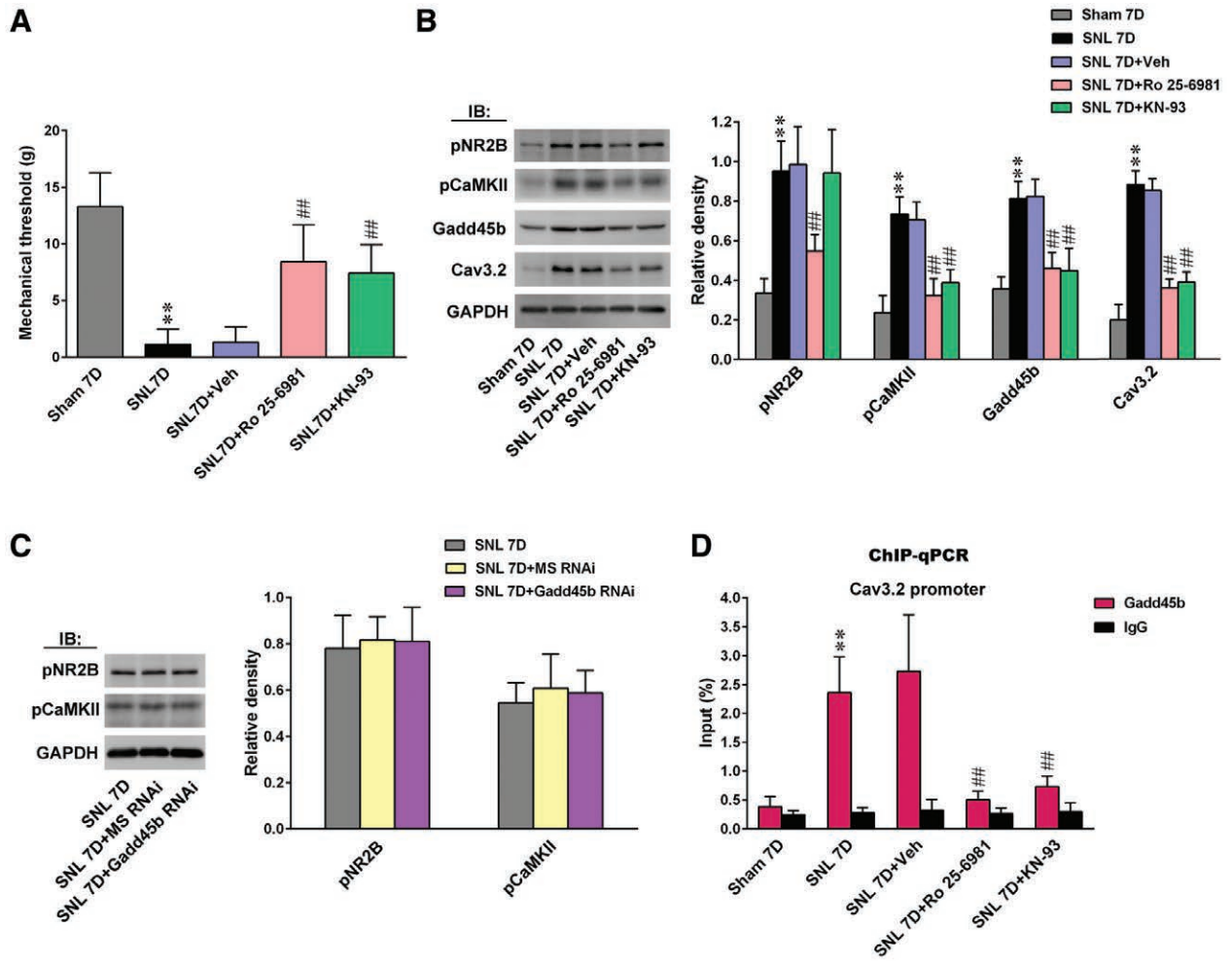

Fig. 6. Spinal nerve ligation provokes spinal NR2B-bearing NMDAR or calmodulin-dependent protein kinase II–dependent growth arrest and DNA-damage–inducible protein 45β (Gadd45β) activation to alter *voltage-dependent T-type calcium channel 3.2 subunit* ($Ca_V3.2$) expression. (*A*) Injection of Ro 25–6981 (day 7 after spinal nerve ligation [SNL 7D] + Ro 25–6981; an NR2B-bearing *N*-methyl-D-aspartate receptor antagonist; 100 nM, 10 μl; once daily on days 3 to 6 after SNL) and KN-93 (SNL 7D + KN-93; a calmodulin-dependent protein kinase II [CaMKII] antagonist; 50 nM, 10 μl; once daily on days 3 to 6 after SNL) both significantly reversed the SNL-decreased mechanical threshold on day 7. ***P* < 0.01 *versus* Sham 7D. ##*P* < 0.01 *versus* SNL 7D. n = 7. (*B*) Representative Western blotting and statistical analysis (normalized to glyceraldehyde 3-phosphate dehydrogenase protein [GAPDH]) demonstrating that SNL (SNL 7D) increased phosphorylated NR2B (pNR2B), phosphorylated CaMKII (pCaMKII), Gadd45β, and $Ca_V3.2$ expression in the ipsilateral dorsal horn on day 7 postoperation, which were all reversed by spinal daily administration of Ro 25–6981 (SNL 7D + Ro 25–6981; 100 nM, 10 μl; once daily on days 3 to 6 after SNL). KN-93 (SNL 7D + KN-93; 50 nM, 10 μl; once daily on days 3 to 6 after SNL) reversed the SNL-increased pCaMKII, Gadd45β, and $Ca_V3.2$ expression in the ipsilateral dorsal horn on day 7 postoperation without affecting the abundance of pNR2B. ***P* < 0.01 *versus* Sham 7D. ##*P* < 0.01 *versus* SNL 7D. n = 6. (*C*) Spinal administration of neither Gadd45β mRNA–targeting siRNA (SNL 7D + Gadd45β RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL) nor missense siRNA (SNL 7D + MS RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL) affected SNL–up-regulated pNR2B and pCaMKII expression in the ipsilateral dorsal horn on day 7 postoperation. (*D*) Chromatin immunoprecipitation (ChIP) quantitative reverse-transcription polymerase chain reaction analysis showed that SNL (SNL 7D) increased the binding of Gadd45β to the $Ca_V3.2$ promoter region in the ipsilateral dorsal horn on day 7 after operation, which was reversed by intrathecal application of Ro 25–6981 (SNL 7D + Ro 25–6981; 100 nM, 10 μl; once daily on days 3 to 6 after SNL) and KN-93 (SNL 7D + KN-93; 50 nM, 10 μl; once daily on days 3 to 6 after SNL). ***P* < 0.01 *versus* Sham 7D. ##*P* < 0.01 *versus* SNL 7D. n = 6. Data represent mean ± SD. IB = immunoblotting; Sham = sham operation; Veh = vehicle.

in the ipsilateral dorsal horn and DRG on day 7 after the operation (fig. 2, E and F; from 0.58 ± 0.06 to 0.28 ± 0.05 and from 0.63 ± 0.12 to 0.48 ± 0.07; n = 6). Furthermore, the SNL-enhanced Gadd45β expression in the DRG was markedly less than that seen the dorsal horn (fig. 2G; spinal cord, 2.97 ± 0.54; DRG, 1.67 ± 0.32; n = 6). Additionally, we found that after administration of Gadd45β mRNA–targeted siRNA for 4 days, SNL-associated allodynia gradually resolved by day 17 after the operation (fig. 2H; 14.12 ± 2.47, 2.62 ± 1.76, 1.14 ± 0.49, 5.42 ± 2.50, 7.85 ± 3.84, 7.57 ± 3.55,

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5.71 ± 2.13, 4.20 ± 2.53, 2.68 ± 1.25, 1.51 ± 0.62, 1.22 ± 0.63, and 0.69 ± 0.41; n = 7). Taken together, these data suggest that spinal Gadd45β significantly contributes to SNL-associated nociceptive hypersensitivity.

Knockdown of Spinal Gadd45β Expression Ameliorates SNL-decreased Rearing Activity

Rearing behavior were recorded on day 7 postoperation from animals that received sham operation and SNL with or without administration with Gadd45β mRNA–targeted siRNA (fig. 3A). Compared to the sham operation, SNL significantly decreased the number and duration of rearing (fig. 3B–D). Furthermore, intrathecal application of a Gadd45β mRNA–targeted siRNA significantly increased the number and duration of rearing compared to the SNL group (fig. 3B–D). This result indicated that SNL-modified rearing behaviors could be reversed by focal knockdown of spinal Gadd45β expression.

Spinal Gadd45β Promotes $Ca_V3.2$ Expression after SNL

In the CNS, Gadd45β has been shown to reactivate methylation-silenced target loci.[3] As the transcription of the $Ca_V3.2$ gene is thought to be regulated by the methylation of CpG sites,[8] and spinal $Ca_V3.2$ channels are proposed to mediate neuropathic hypersensitivity,[35] we wondered whether spinal Gadd45β participates in the development of neuropathic allodynia by promoting $Ca_V3.2$ expression. To characterize the role of $Ca_V3.2$ in neuropathic allodynia, we first examined whether antagonism of spinal $Ca_V3.2$ would affect this process using L-ascorbic acid (a selective $Ca_V3.2$ antagonist).[36] Seven days after SNL, intrathecal injections of L-ascorbic acid (10, 30, or 100 μM; 10 μl each) dose dependently increased the withdrawal threshold of the ipsilateral hind paw 1 to 6 h after injection (fig. 4A; 10 μM: 2.77 ± 1.64, 4.85 ± 1.95, 4.00 ± 2.00, 2.77 ± 1.16, 1.94 ± 1.07, and 1.85 ± 1.48; 30 μM: 4.00 ± 2.30, 7.14 ± 3.02, 6.00 ± 2.58, 5.42 ± 2.22, 3.71 ± 2.13, and 4.00 ± 2.31; 100 μM: 5.14 ± 1.57, 7.71 ± 1.38, 7.42 ± 1.51, 6.28 ± 2.42, 5.71 ± 2.69, and 5.14 ± 1.06; n = 7). Moreover, L-ascorbic acid injection (100 μM, 10 μl) did not affect the response of the contralateral hind paw 2 h after injection, a time point at which L-ascorbic acid displays its maximal analgesic effects (fig. 4B). We next examined whether overexpression of Gadd45β in naive rats is sufficient to enhance the $Ca_V3.2$ expression that underlies the development of allodynia. Intriguingly, intrathecal injections of lentiviral vector (LV) expressing *Gadd45β*, but not a control vector, into naive rats resulted in a decreased withdrawal threshold (Naive, 12.42 ± 6.42; control LV, 11.85 ± 4.45; *Gadd45β* LV, 3.28 ± 1.70; n = 7) associated with increases in Gadd45β and $Ca_V3.2$ protein levels in the ipsilateral dorsal horn (0.58 ± 0.08 and 0.76 ± 0.14; n = 6) on day 14 after treatment (fig. 4, C and D). Next, we explored whether the transcription or protein expression of $Ca_V3.2$ in the ipsilateral dorsal horn and DRG was altered after SNL and if these changes were affected by knockdown of Gadd45β. In ipsilateral dorsal horn and DRG samples harvested on day 7 postoperation, we found that the mRNA (2.08 ± 0.15 and 1.37 ± 0.06; n = 5) and protein (0.81 ± 0.07 and 0.71 ± 0.14; n = 6) levels of $Ca_V3.2$ were both significantly increased in the SNL group compared with the sham-operated group (fig. 4E–J). Conversely, the SNL-induced up-regulation of $Ca_V3.2$ mRNA and protein levels in the ipsilateral dorsal horn and DRG was significantly decreased by intrathecal administration of a Gadd45β mRNA–targeted siRNA (5 μg, 10 μl; spinal cord, 1.09 ± 0.56 and 0.45 ± 0.10; DRG, 1.10 ± 0.11 and 0.55 ± 0.07; n = 5 or 6). Interestingly, the magnitude of the changes in mRNA and protein expression in the DRG was markedly less than that seen in the dorsal horn (mRNA, 2.06 ± 0.16 and 1.47 ± 0.28; protein, 3.31 ± 1.46 and 1.54 ± 0.32; n = 6). ChIP quantitative reverse-transcription polymerase chain reaction analysis of ipsilateral dorsal horn samples further demonstrated that SNL significantly increased the binding of Gadd45β to the $Ca_V3.2$ promoter on day 7 after the operation (2.08 ± 0.42; n = 6), and this was reversed by intrathecal application of a Gadd45β mRNA–targeted siRNA (fig. 4K; 5 μg, 10 μl; 0.68 ± 0.15; n = 6). Taken together, these findings suggest that SNL promotes Gadd45β expression, which in turn promotes $Ca_V3.2$ transcription and expression in the dorsal horn, to mediate nociceptive hypersensitivity postneuropathic injury.

Gadd45β Mediates SNL-induced Enhancement of $Ca_V3.2$ Channel–mediated Currents

We next studied whether genetic knockdown of Gadd45β could reverse the SNL-induced enhancement of $Ca_V3.2$ currents in spinal dorsal horn neurons. To test this hypothesis, we analyzed the relative contribution of $Ca_V3.2$-dependent current to the total T-type calcium channel current in dorsal horn neurons in spinal slices dissected on day 7 postoperation. When compared with sham-operated animals (Sham 7D), SNL significantly increased total T-type current compared to dorsal horn neurons from sham-operated animals (Sham 7D) (fig. 5A). This effect was attenuated by administration of a Gadd45β mRNA–targeted siRNA (SNL 7D + Gadd45β RNAi; fig. 5A) but not a missense siRNA (SNL 7D + MS RNAi). Application of 300 μM L-ascorbic acid largely abolished the SNL-induced enhancement of currents. We isolated the current–voltage (I–V) relationship of the $Ca_V3.2$-dependent current by subtracting the current observed in the presence of L-ascorbic acid from the total T-type calcium channel current. Knockdown of spinal Gadd45β expression significantly decreased $Ca_V3.2$-dependent current compared to the same current in neurons from rats that received SNL (fig. 5, B and C; Sham 7D, 3.31 ± 1.46; SNL 7D, 22.05 ± 2.40; SNL 7D + MS RNAi, 19.30 ± 4.15; SNL 7D + Gadd45β RNAi, 10.73 ± 1.39; n = 6) as evidenced by the I–V relationship and peak I–V values (fig. 5, B and C; Sham 7D, 3.31 ± 1.46; SNL 7D, 22.05 ± 2.40; SNL 7D + MS RNAi, 19.30 ± 4.15; SNL 7D + Gadd45β RNAi, 10.73 ± 1.39; n = 6). These results imply that the functional significance of spinal Gadd45β in the development of neuropathic allodynia is its impact on $Ca_V3.2$ in dorsal horn neurons.

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NR2B-containing NMDAR or CaMKII Signaling Promotes Gadd45β-mediated Spinal $Ca_V3.2$ Expression after SNL

A previous study demonstrated that an NMDAR agonist leads to the activation of CaMK, which up-regulates Gadd45β expression, resulting in the induction of target gene transcription.[5] NR2B-containing NMDAR or CaMKII signaling has also been linked to the machinery underlying neuropathic pain development,[13] and we previously demonstrated that NR2B-containing NMDAR phosphorylation in the spinal neuraxis is critical for SNL-induced behavioral allodynia.[14] In addition, calcium ion-dependent CaMKII signaling epigenetically regulates mechanisms of calcium channel transcription,[17] and $Ca_V3.2$ channels are associated with NMDAR[15] and CaMKII signaling[16] in neurons. Thus, we examined whether NR2B-containing NMDAR phosphorylation contributes to the development of neuropathic allodynia *via* NMDAR or CaMKII signaling and downstream Gadd45β-promoted $Ca_V3.2$ expression. The von Frey test showed that the daily administration of Ro 25–6981 (an NR2B antagonist; 100 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL; 9.28 ± 3.09; n = 7) and KN-93 (a CaMKII antagonist; 50 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL; 8.71 ± 3.30; n = 7), but not a vehicle solution (10 μl), reversed the decrease in withdrawal threshold of the ipsilateral hind paw normally apparent at 3 h after SNL 7D (fig. 6A; 1.21 ± 0.71; n = 7*)*. On day 7 postoperation, we found that SNL predictably increased the abundance of pNR2B, pCaMKII, Gadd45β, and $Ca_V3.2$ in the ipsilateral dorsal horn (0.95 ± 0.15, 0.73 ± 0.08, 0.81 ± 0.08, and 0.88 ± 0.06; n = 6), and these effects were markedly reversed by daily intrathecal injection of Ro 25–6981 injection (fig. 6B; 100 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL; 0.54 ± 0.08, 0.32 ± 0.08, 0.45 ± 0.08, and 0.36 ± 0.04; n = 6). Moreover, treating animals that received SNL with KN-93 (50 nM, 10 μl; daily from days 3 to 6 after SNL) also significantly reversed SNL-enhanced protein expression (0.38 ± 0.06, 0.44 ± 0.11, and 0.38 ± 0.05; n = 6), with the exception of pNR2B (0.94 ± 0.21; n = 6). Importantly, intrathecal administration of neither a Gadd45β mRNA–targeted siRNA (5 μg, 10 μl) nor a missense siRNA (10 μl) affected SNL-mediated pNR2B or pCaMKII expression (fig. 6C). These results suggest that pNR2B or CaMKII signaling functions upstream of Gadd45β-regulated $Ca_V3.2$ expression in mediating SNL-induced neuropathic allodynia. Finally, based on the above findings, we examined whether the SNL-induced binding of Gadd45β to the $Ca_V3.2$ promoter was affected by intrathecal application of Ro 25–6981 or KN-93. In samples taken from the ipsilateral dorsal horn on day 7 after the operation, we observed an SNL-induced increase in Gadd45β binding to the $Ca_V3.2$ promoter (2.35 ± 0.62; n = 6) that was significantly reversed by intrathecal administration with Ro 25–6981 (100 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL; 0.50 ± 0.15; n = 6) and KN-93 (fig. 6D; 50 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL; 0.73 ± 0.18; n = 6). In addition, immunohistochemical analysis revealed that SNL, but not a sham operation, enhanced the levels of pNR2B (91 ± 16), pCaMKII (60 ± 9), Gadd45β (64 ± 12), and pNR2B, pCaMKII, or Gadd45β triple-labeled immunoreactivity (32 ± 20) in the ipsilateral dorsal horn on day 7 postoperation, and this effect was also reversed by spinal administration of Ro 25–6981 (fig. 7A; 100 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL; 25 ± 12, 28 ± 11, 24 ± 8, and 10 ± 5; n = 7). Spinal administration of KN-93 (50 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL) ameliorated the SNL-induced increase in the levels of pCaMKII (25 ± 9), Gadd45β (24 ± 7), and pNR2B, pCaMKII, or Gadd45β (9 ± 5) triple-labeled immunoreactivity in the ipsilateral dorsal horn on day 7 postoperation but did not affect the SNL-induced increase in the level of spinal pNR2B expression (87 ± 21; n = 7). Taken together, these data suggest that SNL-induced spinal NR2B phosphorylation activates Gadd45β-mediated $Ca_V3.2$ expression *via* NMDAR or CaMKII signaling.

SNL-activated Spinal NR2B, CaMKII, or Gadd45β Signaling Promotes Demethylation of the $Ca_V3.2$ Promoter via Conversion of 5-Formylcytosine and 5-Carboxylcytosine to 5-Cytosine

Gadd45β has been implicated in active DNA demethylation within target promoters, which reactivates methylation-silenced target loci.[3] Gadd45 has been demonstrated to promote active DNA demethylation by recognizing and excising 5-formylcytosine and/or 5-carboxylcytosine,[24] which are DNA demethylation intermediates, thereby resulting in reversion to unmodified 5-cytosine for complete DNA demethylation.[19,20] To extend our findings, we first assayed the enrichment of unmodified 5-cytosine at the $Ca_V3.2$ promoter after SNL. We found that SNL increased the amount of unmodified 5-cytosine at the $Ca_V3.2$ promoter (fig. 8A; 2.16 ± 0.42; n = 6) and that this effect was reduced by the administration of Ro 25–6981 (100 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL; 0.59 ± 0.10; n = 6), KN-93 (50 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL; 0.71 ± 0.16; n = 6), or a Gadd45β mRNA–targeted siRNA (5 μg, 10 μl; daily for 4 days from days 3 to 6 after SNL; 0.37 ± 0.11; n = 6). Furthermore, we found that SNL significantly increased the global levels of 5-formylcytosine and 5-carboxylcytosine in the ipsilateral dorsal horn on day 7 postoperation (fig. 8, B and C; 9.22 ± 2.29 and 7.39 ± 1.99; n = 6). Notably, intrathecal administration of Ro 25–6981 (100 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL), KN-93 (50 nM, 10 μl; daily for 4 days from days 3 to 6 after SNL), or a Gadd45β mRNA–targeted siRNA (5 μg, 10 μl; daily for 4 days from days 3 to 6 after SNL) further enhanced the SNL-induced increase in the global levels of 5-formylcytosine (15.88 ± 2.36, 16.63 ± 2.79, and 15.72 ± 3.94; n = 6) and 5-carboxylcytosine (12.84 ± 1.85, 12.92 ± 2.86, and 12.82 ± 3.59; n = 6) in the ipsilateral dorsal horn. These results imply that NR2B-containing NMDAR or CaMKII signaling promotes Gadd45β-dependent DNA

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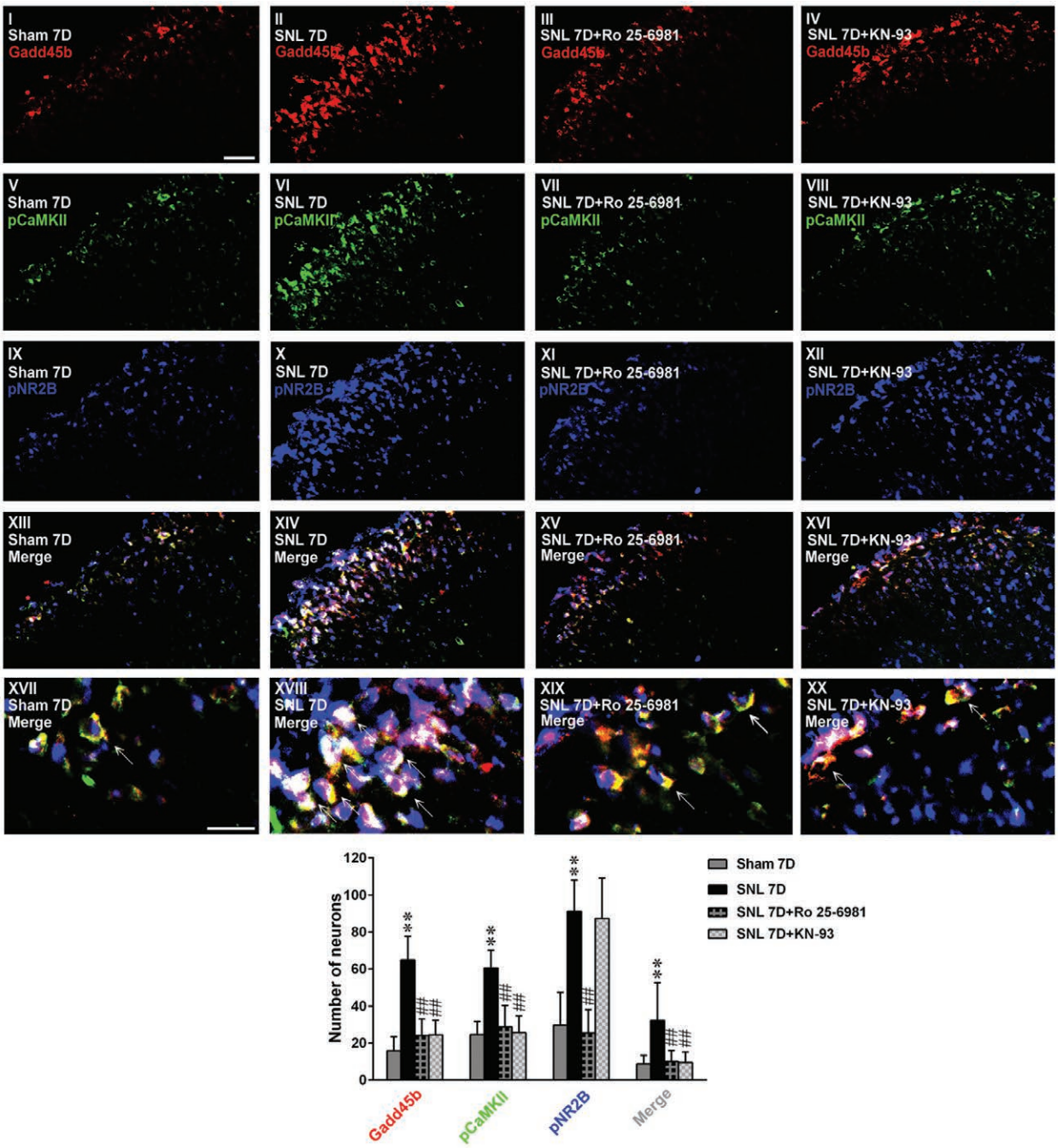

Fig. 7. Spinal nerve ligation activates spinal NR2B-bearing NMDAR or calmodulin-dependent protein kinase II signaling and downstream growth arrest and DNA-damage–inducible protein 45β (Gadd45β) to mediate behavioral allodynia. Image analysis demonstrated that, compared with sham operation (Sham 7D), spinal nerve ligation (SNL 7D) markedly increased phosphorylated NR2B-bearing *N*-methyl-D-aspartate receptor (pNR2B, *red*), phosphorylated calmodulin-dependent protein kinase II (pCaMKII, *green*), Gadd45β (*blue*), and pNR2B, pCaMKII, or Gadd45β triple-labeled (merge, *white*) immunofluorescence in the ipsilateral dorsal horn on day 7 postoperation, which were all reversed by spinal administration of Ro 25–6981 (SNL 7D + Ro 25–6981; a selective NR2B receptor antagonist; 100 nM, 10 μl; once daily on days 3 to 6 after SNL). KN-93 (SNL 7D + KN-93; a CaMKII antagonist; 50 nM; once daily on days 3 to 6 after SNL) reversed the SNL-increased pCaMKII, Gadd45β expression, and pNR2B, pCaMKII, or Gadd45β triple-labeled (*white*) without affecting the levels of pNR2B. *Scale bar* = 50 μm. *Thickness* = 50 μm. ***P* < 0.01 *versus* Sham 7D. ##*P* < 0.01 *versus* SNL 7D. n = 7. Data represent mean ± SD.

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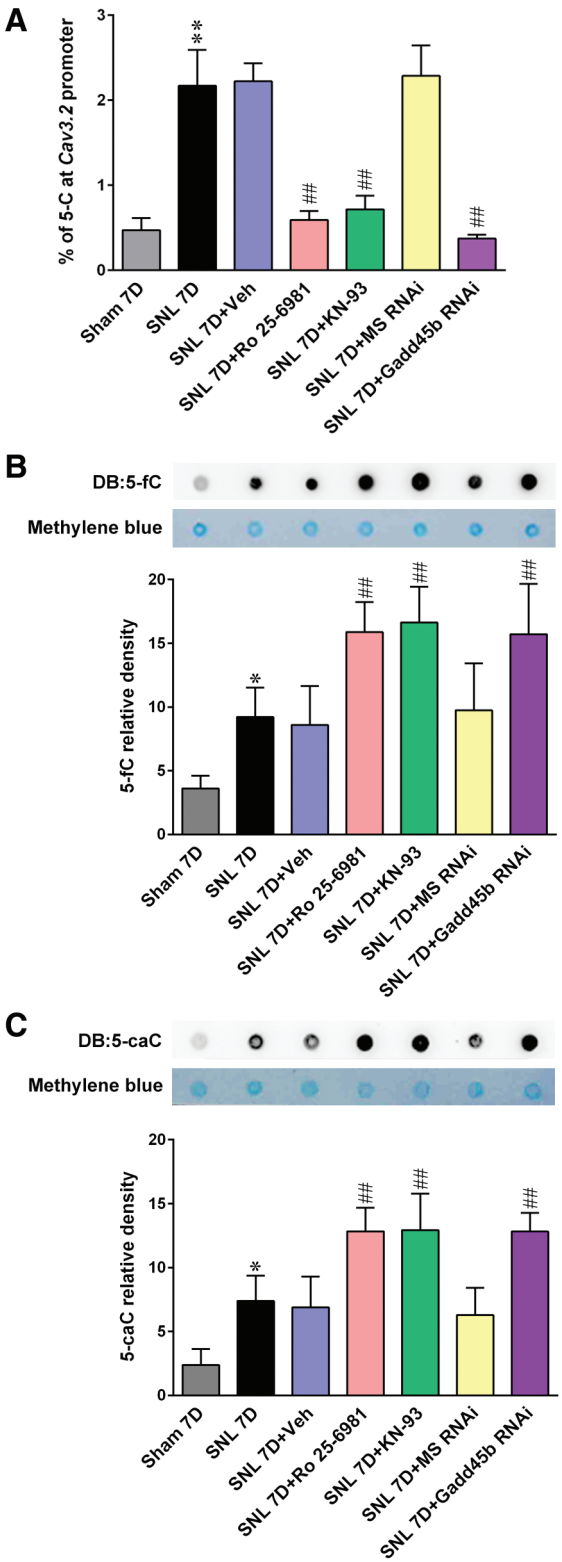

Fig. 8. Spinal nerve ligation-provoked spinal NR2B-bearing NMDAR or calmodulin-dependent protein kinase II or growth arrest and DNA-damage–inducible protein 45β (Gadd45β) or *voltage-dependent T-type calcium channel 3.2 subunit* ($Ca_V3.2$) signaling promotes 5-formylcytosine (5-fC) or 5-cytosine and 5-carboxylcytosine (5-caC) or 5-cytosine conversion. (*A*) Representative statistical analysis revealing that the spinal nerve demethylation at the $Ca_V3.2$ promoter through the conversion of 5-carboxylcytosine and 5-formylcytosine to 5-cytosine after SNL.

Discussion

In this study, we verified the pivotal role of spinal Gadd45β in nociceptive hypersensitivity after neuropathic insult and revealed that NR2B-containing NMDAR or CaMKII signaling facilitates Gadd45β-mediated DNA demethylation at the $Ca_V3.2$ promoter in dorsal horn neurons to underlie the development of neuropathic allodynia. Our conclusion is based on results showing that neuropathic injury-induced allodynia is accompanied by the time-dependent up-regulation of Gadd45β expression in ipsilateral dorsal horn neurons. Knockdown of Gadd45β expression not only attenuated SNL-induced allodynia but also inhibited the SNL-induced enhancement of $Ca_V3.2$ expression, $Ca_V3.2$-dependent currents, and Gadd45β binding to the $Ca_V3.2$ promoter in the dorsal horn on day 7 postoperation. Conversely, overexpression of Gadd45β in naive rats provoked allodynia and up-regulated spinal $Ca_V3.2$ expression. Through the daily intrathecal administration of NR2B- or CaMKII-specific antagonists or through a Gadd45β mRNA–targeted siRNA in allodynic rats, we found that NR2B-containing NMDAR or CaMKII signaling contributes to the development of allodynia *via* the potentiation of Gadd45β-mediated $Ca_V3.2$ gene demethylation in the dorsal horn. Additionally, daily intrathecal application of Ro 25–6981, KN-93, or a

Fig. 8. (*Continued*). ligation (SNL)–induced increased in unmodified 5-cytosine enrichment at the $Ca_V3.2$ promoter on day 7 postoperation was reversed by intrathecal administration of Ro 25–6981 (SNL 7D + Ro 25–6981; an NR2B-bearing *N*-methyl-D-aspartate receptor antagonist; 100 nM, 10 μl; once daily on days 3 to 6 after SNL), KN-93 (SNL 7D + KN-93; a calmodulin-dependent protein kinase II [CaMKII] antagonist; 50 nM, 10 μl; once daily on days 3 to 6 after SNL) and Gadd45β mRNA–targeting siRNA (SNL 7D + Gadd45β RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL). Missense siRNA (MS RNAi; 5 μg, 10 μl). $^{**}P < 0.01$ *versus* Sham 7D. ##$P < 0.01$ *versus* SNL 7D. n = 6. (*B*, *C*) Representative dot blot analysis (*top*) showing that SNL (SNL 7D) increased global 5-caC and 5-fC levels in the ipsilateral, but not contralateral, dorsal horn on day 7 after operation compared with sham operation (Sham 7D). Ro 25–6981 (SNL 7D + Ro 25–6981; 100 nM, 10 μl; once daily on days 3 to 6 after SNL), KN-93 (SNL 7D + KN-93; 50 nM, 10 μl; once daily on days 3 to 6 after SNL) and Gadd45β mRNA–targeting siRNA (SNL 7D + Gadd45β RNAi; 5 μg, 10 μl; once daily on days 3 to 6 after SNL) further enhanced the SNL-increased global levels of 5-caC and 5-fC in the ipsilateral dorsal horn. Methylene blue staining (*middle*) was used to validate equal DNA loading. Statistical analysis (*bottom*) of the relative intensity of 5-fC and 5-caC in the dot blot analysis in both groups. $^{*}P < 0.05$ *versus* Sham 7D. ##$P < 0.01$ *versus* SNL 7D. n = 6. Data represent mean ± SD. DB = dot blotting; Veh = vehicle.

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Gadd45β mRNA–targeted siRNA also reversed the SNL-induced enrichment of 5-cytosine at the $Ca_V3.2$ promoter, accompanied by an increase in SNL-induced 5-formylcytosine and 5-carboxylcytosine levels.

Moreover, intrathecal administration of a Gadd45β mRNA–targeted siRNA not only ameliorated SNL-associated allodynia but also reduced SNL effects on rearing activity. Though rearing is used as a measure of anxiety and general exploratory behavior, some studies[37,38] suggest rearing activity is a pain-related behavior because it is sensitive to analgesics. Hence, despite our inability to use rearing as a specific index of nonevoked pain behavior, our data at least revealed reduced expression of spinal Gadd45β ameliorated a behavioral change potentially related to the sensory and/or emotional consequences of nerve injury. Altogether, these results imply NR2B, CaMKII, or Gadd45β signaling promotes $Ca_V3.2$ promoter demethylation *via* the conversion of 5-formylcytosine and 5-carboxylcytosine to unmethylated 5-cytosine. To the best of our knowledge, our study is the first report that Gadd45β mediates DNA demethylation–dependent epigenetic regulation of the $Ca_V3.2$ promoter during neuropathic insult. Therefore, these findings are potentially of considerable benefit to the treatment of chronic neuropathic allodynia.

On the other hand, studies have demonstrated intrathecal delivery of reagents is not only restricted to the dorsal horn neurons at the injection site but also affect DRG neurons.[39] In our study, although the relative magnitude of the changes compared to the sham-operated animals was markedly smaller in the DRG compared with the dorsal horn, SNL up-regulated Gadd45β expression in both the dorsal horn and the DRG, and this was reversed by intrathecal administration of a Gadd45β mRNA–targeted siRNA, indicating Gadd45β in both the dorsal horn and the DRG contributes crucially to the neuropathic allodynia. Therefore, we specifically focused on neuropathic injury-induced changes in the dorsal horn in the current study and observed enhanced expression of Gadd45β and $Ca_V3.2$ and phosphorylation of CaMKII and NR2B in the dorsal horn after SNL. More importantly, electrophysiologic recordings from spinal slices revealed a Gadd45β-dependent enhancement of $Ca_V3.2$ activity caused by neuropathic injury. Collectively, we conclude Gadd45β-associated plasticity in the dorsal horn plays a role in the development of neuropathic allodynia. Nevertheless, the potential contribution of Gadd45β in the DRG to neuropathic allodynia requires further study.

Growth arrest and DNA-damage–inducible protein 45 family members (*i.e.*, Gadd45a, Gadd45β, and Gadd45g) are drivers of active demethylation.[1] Among them, Gadd45β was recently suggested to be a regulator of plasticity-related genes.[13] Gadd45β is required for the activity-induced DNA demethylation of specific promoters and the expression of corresponding genes critical for neurogenesis and neural activity in hippocampal neurons.[3] Consistently, Gadd45β expression is increased in the mouse hippocampus after context-exposure learning[40] and fear conditioning.[41] In agreement with these studies linking Gadd45β to forms of activity-dependent plasticity in the brain, our results revealed Gadd45β-mediated demethylation mechanisms also participate in chronic neuropathic allodynia and govern spinal neural plasticity that encodes behavioral hypersensitivity to noxious stimuli. Our findings are supported by work investigating neuropathic nociceptive hypersensitivity mechanisms, which suggests that nociceptive-associated plasticity in spinal neurons relies on molecular processes that are similar to those underlying associative learning, particularly learning- and memory-associated areas such as the hippocampus.[42]

Studies have proposed possible therapeutic effects of NMDAR-specific antagonists for neuropathic pain relief.[43] Our previous reports suggest spinal NR2B-containing NMDARs play an important role in the central sensitization underlying neuropathic allodynia.[14,27] Moreover, $Ca_V3.2$, a T-type Ca^{2+} channel, is known to be involved in spinal central sensitization–associated synaptic plasticity,[35,44] making it another novel therapeutic target for neuropathic allodynia. Notably, evidence suggests that NMDAR antagonists reverse $Ca_V3.2$ channel–mediated transmission at central synapses.[44] In addition, T-type Ca^{2+} channels coactivated with NR2B-NMDARs induce long-term potentiation at synapses in the thalamoreticular area,[45] indicating spinal NR2B or $Ca_V3.2$ signaling may play a critical role in neuropathic allodynia. A recent study showed that genetic Gadd45β depletion inhibited Ca^{2+}-induced gene transcription, suggesting that Gadd45β is necessary for this process.[46] The application of agonists specific to the NMDAR, a known Ca^{2+}-permeable ion channel, results in membrane depolarization and therefore increases Gadd45β expression in cultured neurons.[3,41] Furthermore, neurons lacking Gadd45β exhibit reduced DNA demethylation and thus fail to activate crucial neurogenic genes,[3,47] which is essential for the spinal plasticity that underlies persistent pain.[6,7] Consistently, we found that NR2B-containing NMDAR phosphorylation activated Gadd45β-mediated DNA demethylation, which is required for the reactivation of the methylation-silenced $Ca_V3.2$ gene in neuropathic nociceptive hypersensitivity. Our findings provide evidence for the novel concept that the phosphorylation of spinal NR2B-containing NMDAR activates $Ca_V3.2$ expression and the development of neuropathic allodynia *via* Gadd45β-mediated DNA demethylation at the $Ca_V3.2$ promoter.

Calmodulin-dependent protein kinase II contributes to the development of central sensitization by phosphorylating various proteins, including neuronal membrane receptors and intracellular transcription factors.[48] Calmodulin-dependent protein, acting through CaMKII, can regulate the activation of the transcription factor c-Rel.[49] Moreover, a recent study demonstrated that Gadd45β-mediated DNA demethylation in hippocampal neurons was blocked of the c-Rel knockout mice,[50] indicating that Gadd45β is a potential downstream target of c-Rel. Thus, Gadd45β activation in

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neurons may be dependent on the well-characterized CaMKII or c-Rel signaling cascade, and the interaction between CaMKII, c-Rel, and Gadd45β in neuropathic pain requires further study to be elucidated.

Tet proteins catalyze the conversion of 5-mC to 5-hmC, and the subsequent oxidation to 5-formylcytosine and 5-carboxylcytosine in what is known as oxidative demethylation, to reactivate methylation-silenced genes at specific genomic loci.[51] Tet overexpression increases 5-hmC but decreases 5-mC enrichment at CpG sites in specific promoters and thus enhances transcription and expression.[52] Formalin injection into the mouse hind paw enhances spinal Tet expression,[22] which further promotes the DNA demethylation to participate in acute inflammatory pain by converting 5-mC to 5-hmC. Additionally, increasing removal of 5-formylcytosine and 5-carboxylcytosine promotes DNA demethylation in Tet overexpressing cells.[24] Consistently, our study found that neuropathic injury enhanced global 5-formylcytosine and 5-carboxylcytosine levels in the dorsal horn. This suggests that SNL promotes DNA demethylation to reactivate methylation-silenced $Ca_V3.2$ *via* Tet-dependent catalysis of the conversion of 5-mC to 5-hmC, as well as subsequent oxidation to 5-formylcytosine and 5-carboxylcytosine. These DNA demethylation intermediates are subject to nucleotide excision repair, resulting in a reversion back to unmodified cytosine and thereby the reactivation of methylation-silenced genes at specific genomic loci.[19–21] Intermediates such as 5-formylcytosine and 5-carboxylcytosine are recognized and excised by Gadd45, which results in reversion back to unmodified cytosine, thus completing the active demethylation process.[24] In this study, we observed that SNL significantly increased global 5-formylcytosine and 5-carboxylcytosine levels in the ipsilateral dorsal horn on day 7 postoperation and that these levels were further increased by intrathecal administration of a Gadd45β mRNA–targeted siRNA. SNL also enhanced unmodified 5-cytosine enrichment at the $Ca_V3.2$ promoter, which was reduced by intrathecal administration of a Gadd45β mRNA–targeted siRNA. Altogether, these findings characterize the Gadd45β-dependent demethylation of the $Ca_V3.2$ promoter, which restores unmodified 5-cytosine by excising increased 5-formylcytosine and 5-carboxylcytosine to mediate the spinal plasticity that underlies nociceptive hypersensitivity. However, the role of Tet family proteins in Gadd45β-related demethylation signaling in neuropathic nociceptive hypersensitivity warrants further study.

Nevertheless, there are limitations to our study. First, to direct use NMDAR- or $Ca_V3.2$-targeted antagonists in clinical scenarios for pain relief is quite limited because both NMDARs and $Ca_V3.2$ are widely expressed in the human body. Pharmacologic antagonism of these receptors, even if restricted to the CNS, could result in significant side effects as NMDAR-selective antagonists cause serious psychotomimetic effects[53] and the down-regulation of $Ca_V3.2$ expression is linked to neurologic diseases such as amyotrophic lateral sclerosis[54] and absence epilepsy.[55] Moreover, in this study, we pharmacologically antagonized $Ca_V3.2$ activity using L-ascorbic acid. In addition to blocking $Ca_V3.2$, L-ascorbic acid is shown to function as an antioxidant.[56,57] Thus, whether the amelioration of allodynia by L-ascorbic acid is at least partially attributable to its antioxidative effects warrants further study. Additionally, Gadd45β promotes DNA demethylation at the regulatory regions of brain-derived neurotrophic factor (BDNF) gene,[3,4] which is accompanied by transcriptional activation of BDNF[1,5]; numerous studies have linked BDNF transcription to spinal plasticity underlying neuropathic pain.[7] In the current study, we demonstrated a neuropathic injury–induced spinal Gadd45β expression accompanied by allodynia. Because BDNF is linked to nociception-associated plasticity, the possibility that BDNF acts as the downstream of Gadd45β to mediate neuropathic hypersensitivity through Gadd45β–dependent demethylation of the BDNF gene cannot be excluded.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Peng: Department of Medicine, Mackay Medical College, No.46, Sec. 3, Zhongzheng Rd, Sanzhi Dist, New Taipei 25245, Taiwan. 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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