Up-regulation of Cavβ3 Subunit in Primary Sensory Neurons Increases Voltage-activated Ca2+ Channel Activity and Nociceptive Input in Neuropathic Pain*

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Background: We determined how nerve injury affects calcium channel β (Cavβ) subunits in neuropathic pain.

Results: Nerve injury increases Cavβ3 expression level, and Cavβ3 knockdown reduces increased calcium channel activity in primary sensory neurons and pain hypersensitivity.

Conclusion: Cavβ3 subunit up-regulation augments calcium channel activity in neuropathic pain.

Significance: Understanding molecular changes in sensory neurons is important for developing new treatments for neuropathic pain.

High voltage-activated calcium channels (HVACCs) are essential for synaptic and nociceptive transmission. Although blocking HVACCs can effectively reduce pain, this treatment strategy is associated with intolerable adverse effects. Neuronal HVACCs are typically composed of α, β (Cavβ), and α,δ subunits. The Cavβ subunit plays a crucial role in the membrane expression and gating properties of the pore-forming α subunit. However, little is known about how nerve injury affects the expression and function of Cavβ subunits in primary sensory neurons. In this study, we found that Cavβ3 and Cavβ4 are the most prominent subtypes expressed in the rat dorsal root ganglion (DRG) and dorsal spinal cord. Spinal nerve ligation (SNL) in rats significantly increased mRNA and protein levels of the Cavβ3, but not Cavβ2, subunit in the DRG. SNL also significantly increased HVACC currents in small DRG neurons and monosynaptic excitatory postsynaptic currents of spinal dorsal horn neurons evoked from the dorsal root. Intrathecal injection of Cavβ3-specific siRNA significantly reduced HVACC currents in small DRG neurons and the amplitude of monosynaptic excitatory postsynaptic currents of dorsal horn neurons in SNL rats. Furthermore, intrathecal treatment with Cavβ3-specific siRNA normalized mechanical hyperalgesia and tactile allodynia caused by SNL but had no significant effect on the normal nociceptive threshold. Our findings provide novel evidence that increased expression of the Cavβ3 subunit augments HVACC activity in primary sensory neurons and nociceptive input to dorsal horn neurons in neuropathic pain. Targeting the Cavβ3 subunit at the spinal level represents an effective strategy for treating neuropathic pain.

Chronic pain, such as neuropathic pain induced by peripheral nerve injury, leads to prolonged excruciating suffering and a reduced quality of life. However, effective treatment of chronic pain remains a major challenge, with only half of patients receiving adequate pain relief (1). Pain can occur spontaneously or as a result of exposure to mildly painful stimuli (hyperalgesia) or stimuli not normally perceived as painful (alldynia). Our understanding of the mechanisms that sustain chronic neuropathic pain conditions is incomplete. It is likely that neuropathic pain becomes chronic as a result of the plasticity of both peripheral nerves and spinal dorsal horn neurons. Clinical studies of patients with neuropathic pain have indicated that the altered central processing associated with pain is maintained dynamically by ongoing peripheral input (2, 3). However, the molecular mechanisms underlying persistent nociceptive input from primary afferent nerves in neuropathic pain are not fully known.

High voltage-activated calcium channels (HVACCs) are essential for many physiological functions, such as neurotransmitter release, membrane excitability, gene transcription, and synaptic plasticity (4, 5). HVACCs in neurons are typically composed of a pore-forming α subunit (the principal component of HVACCs) and accessory β (Cavβ), α,δ, and possibly γ subunits (5, 6). HVACCs are clarified as the L (Ca1.1–1.4)-type, N (Ca2.2)-type, P/Q (Ca2.1)-type, and R (Ca2.3)-type according to the different biophysical and pharmacological properties of their α subunits (5, 7). The α,δ subunit can augment functional expression of the α subunit (8) and is up-regulated in the dorsal root ganglion (DRG) after nerve injury (9). Another important auxiliary subunit, the Cavβ subunit, is involved in promoting the surface expression of the α subunit and the gating properties of HVACCs (10–12). There are four Cavβ subunits, each with splice variants, encoded by four distinct

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‡ The abbreviations used are: HVACC, high voltage-activated calcium channels; DRG, dorsal root ganglion; EPSC, excitatory postsynaptic current; SNL, spinal nerve ligation.
genes. The Cavβ_{1} subunit appears to be the only Cavβ subunit expressed in the skeletal muscle (13), whereas the Cavβ_{2} subunit is present primarily in the heart (14). The Cavβ_{3} subunit is strongly expressed in smooth muscle and brain, and the Cavβ_{4} is the predominant subunit in cerebellum (14, 15). Mice deficient in Cavβ_{1} or Cavβ_{2} are not viable (16, 17), and knock-out of Cavβ_{2} or Cavβ_{4} results in a wide range of abnormalities (18, 19). Despite the importance of Cavβ subunits in regulating HVACC activity, little is known about the contribution of Cavβ subunits to nerve injury-induced changes in HVACC activity in primary sensory neurons and neuropathic pain.

Therefore, we used a rat model of neuropathic pain to determine how nerve injury affects the expression level of Cavβ subunits in the DRG and dorsal spinal cord. We also determined the contribution of the Cavβ_{3} subunit to increased HVACC activity in DRG neurons and nociceptive input to the spinal cord induced by nerve injury. Our findings indicate that among the four Cavβ subunits in the DRG and spinal cord, Cavβ_{3} and Cavβ_{4} showed the greatest expression. Nerve injury up-regulated the Cavβ_{3} but not Cavβ_{4} subunit in the DRG. Furthermore, siRNA knockdown of Cavβ_{3} greatly reduced increased HVACC activity in the DRG, glutamatergic input to the spinal cord, and pain hypersensitivity caused by nerve injury without impairing normal nociception. Our study highlights the important role of the Cavβ_{3} subunit in increased HVACC activity in primary sensory neurons in neuropathic pain. Therefore, targeting the Cavβ_{3} subunit at the spinal level represents a new strategy for treating neuropathic pain with improved therapeutic profiles.

**EXPERIMENTAL PROCEDURES**

**Neuropathic Pain Model and Intrathecal Cannulation—**Neuropathic pain was induced by SNL in rats, as described previously (20, 21). In brief, male Sprague-Dawley rats initially weighing ∼250 g (Harlan, Indianapolis, IN) were anesthetized with use of 2% isoflurane. The left L5 and L6 spinal nerves were isolated under a surgical microscope and ligated with 4.0 silk suture. Sham surgery was performed in the contralateral side of the same rats or separate rats by using the same procedure except that the spinal nerves were not ligated. In some rats, intrathecal cannulation was performed 7 days after SNL. After rats were anesthetized with isoflurane, catheters (PE-10 polyethylene tubing, 8 cm) were inserted through an incision in the cisternal membrane and advanced caudally so that the tip of each catheter was positioned at the lumbar spinal level (22, 23). The catheters were externalized to the back of the neck and sutured to the musculature and skin at the incision site. All experiments were approved by the Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center and conformed to National Institutes of Health guidelines on the ethical use of animals.

**Nociceptive Behavioral Tests—**To assess mechanical nociception, paw withdrawal thresholds were measured by applying a noxious pressure stimulus using an Ugo Basil Analgesimeter (Varese, Italy). The nociceptive threshold was recorded when rats displayed pain by paw withdrawal or vocalization. The cut-off of 400 g was used to avoid potential tissue injury (22).

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The tactile withdrawal threshold was measured by application of von Frey filaments to both hind paws of SNL rats. Rats were placed individually in plastic cages on a mesh floor and then allowed to acclimate for at least 30 min. A series of calibrated von Frey filaments (Stoelting Co., Wood Dale, IL) were applied perpendicularly to the plantar surface of the hind paw. Brisk withdrawal or paw flinching was considered a positive response. The tactile stimulus that could produce a 50% likelihood of withdrawal was determined by using the “up-down” calculating method (21, 24).

**Isolation of DRG Neurons and Electrophysiological Recording of HVACCs—**Dissociation of DRG neurons was performed essentially as we described previously (25, 26). Rats were anesthetized with isoflurane and then rapidly decapitated. The lumbar segment of the vertebral column was removed quickly. The L5 and L6 DRGs were obtained and transferred into cold DMEM (Invitrogen). After DRGs were minced, they were placed into 5 ml of DMEM containing trypsin (type I, 0.2 mg/ml; Sigma) and collagenase (type I, 1 mg/ml; Sigma). After incubation at 34 °C for 40 min, 10% FBS was used to stop trypsin digestion. Neurons were plated onto a 35-mm culture dish containing poly-L-lysine (50 μg/ml) precoated cover slips. The cells were incubated in fresh medium for at least another hour before they were used for electrophysiological recording of HVACC activity.

HVACC currents were measured using barium as the charge carrier (I_{Ba}) (25, 26). Electrodes with a resistance of 2–3 megohms were pulled from glass capillaries and fire-polished. After establishing the whole-cell configuration, the cell membrane capacitance and series resistance were electronically compensated. Signals were processed with use of an EPC-10 amplifier (HEKA Instruments, Lambrecht, Germany), filtered at 1 kHz, digitized at 10 kHz, and acquired by using the Pulse program (HEKA Instruments). The extracellular solution consisted of 140 mM tetraethylammonium, 2 mM MgCl_{2}, 3 mM BaCl_{2}, 10 mM glucose, and 10 mM HEPES (pH 7.4, osmolarity of 320 mosm). The pipette internal solution consisted of 120 mM CsCl, 1 mM MgCl_{2}, 10 mM HEPES, 10 mM EGTA, 4 mM MgATP, and 0.3 mM NaGTP (pH 7.2, osmolarity of 300 mosm). The voltage-dependent activation of HVACCs was tested at a holding potential of −90 mV and elicited by a series of command potentials from −70 to 50 mV for 150 ms in 10-mV steps (5-s intervals). The voltage-dependent inactivation of HVACCs was assessed by depolarizing cells to a series of prepulse potentials from −90 to 10 mV for 500 ms, followed by a command potential to 0 for 150 ms (25, 26). All experiments were performed at room temperature (∼25 °C).

**Spinal Cord Slice Preparation and Electrophysiological Recording of Synaptic Activity—**After rats were anesthetized with 2–3% isoflurane, the lumbar segment of the spinal cord at the L5/L6 level was removed through laminectomy. The spinal cord tissue was immediately placed in ice-cold sucrose artificial cerebrospinal fluid contained with 95% O_{2} and 5% CO_{2}. The sucrose artificial cerebrospinal fluid contained 234 mM sucrose, 3.6 mM KCl, 1.2 mM MgCl_{2}, 2.5 mM CaCl_{2}, 1.2 mM NaH_{2}PO_{4}, 12 mM glucose, and 25 mM NaHCO_{3}. The tissue was then placed in a shallow groove formed in a gelatin block and glued onto the stage of a vibratome. Transverse spinal cord slices (400 μm)
were cut in the ice-cold sucrose artificial cerebrospinal fluid and preincubated in Krebs’ solution oxygenated with 95% O₂ and 5% CO₂ at 34 °C for at least 1 h before they were transferred to the recording chamber. The Krebs’ solution contained 117 mM NaCl, 3.6 mM KCl, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 11 mM glucose, and 25 mM NaHCO₃ gassed with 95% O₂ and 5% CO₂ at 34 °C for at least 1 h before they were transferred to the recording chamber. The Krebs’ solution contained 117 mM NaCl, 3.6 mM KCl, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 11 mM glucose, and 25 mM NaHCO₃ gassed with 95% O₂ and 5% CO₂.

Each spinal cord slice was placed in a glass-bottomed chamber and continuously perfused with Krebs’ solution at 5.0 ml/min at 34 °C maintained by an in-line solution heater and a temperature controller. The neurons in lamina II (substantia gelatinosa) at the L5 and L6 levels were identified under a fixed-stage microscope (BX50WI; Olympus, Tokyo, Japan) with differential interference contrast/infrared illumination. Lamina II neurons were selected for recording because they primarily receive nociceptive input from peripheral nerve fibers (27, 28).

Recordings of excitatory postsynaptic currents (EPSCs) were performed by using the whole-cell voltage clamp method, as we described previously (23, 29, 30). The impedance of the electrode was 5–10 meghoms when filled with internal solution containing 135 mM potassium gluconate, 5 mM KCl, 2 mM MgCl₂, 25 mM CaCl₂, 1.2 mM NaH₂PO₄, 11 mM glucose, and 25 mM NaHCO₃ gassed with 95% O₂ and 5% CO₂.

Western Blot Analysis—Tissues were sonicated in RIPA buffer and a mixture of protease inhibitors (Sigma). Total protein was extracted by centrifuge at 16,000 × g for 10 min at 4 °C. Equal amounts of proteins (20 μg) were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Immobilon P, Millipore). The blot was probed with anti-Cav β3 (NeuroMab, Davis, CA; 1:1000 dilution), anti-Cav β3 antibody (Santa Cruz Biotechnology; 1:1000 dilution), anti-Cav β4 (NeuroMab; 1:1000 dilution), and anti-GAPDH (Millipore; 1:1000 dilution). ImageJ was used to quantify the band intensities. The amounts of Cav β subunit proteins were normalized by GAPDH (used as an internal control). The mean values of DRGs and spinal cord tissues contralateral to SNL were considered as 1.

Table 2—List of primers used in quantitative PCR

| Gene (accession no.) | Primers |
|----------------------|---------|
| CaV β1 (NM_017346.1) | Fwd: 5’-GGCAAGGCTCCGCGGATAC-3’ Rev: 5’-AGTTGAGGTGTTGCGAC-3’ |
| CaV β1 (NM_053851.1) | Fwd: 5’-GGCAAGGCTCCGCGGATAC-3’ Rev: 5’-AGTTGAGGTGTTGCGAC-3’ |
| CaV β2 (NM_012828.2) | Fwd: 5’-GGCAAGGCTCCGCGGATAC-3’ Rev: 5’-AGTTGAGGTGTTGCGAC-3’ |
| CaV β3 (NM_012828.2) | Fwd: 5’-GGCAAGGCTCCGCGGATAC-3’ Rev: 5’-AGTTGAGGTGTTGCGAC-3’ |
| CaV β4 (NM_012828.2) | Fwd: 5’-GGCAAGGCTCCGCGGATAC-3’ Rev: 5’-AGTTGAGGTGTTGCGAC-3’ |
| GAPDH (NM_012828.2) | Fwd: 5’-GGCAAGGCTCCGCGGATAC-3’ Rev: 5’-AGTTGAGGTGTTGCGAC-3’ |

Using an IQ5 system (Bio-Rad). The sequence primers of the Cav β subunits were listed in Table 2. The mRNA level of the Cav β subunits was calculated using the 2⁻ΔΔCt method and normalized by GAPDH (used as a control). The mean values of DRGs and spinal cord tissues contralateral to SNL were considered as 1.

Double Immunofluorescence Labeling of Cav β3 Subunit and NF200 or Peripherin in the DRG—To determine the cellular distribution of the Cav β3 subunit in the DRG, we performed double immunofluorescence labeling of this Cav β3 subunit with a marker for small neurons (peripherin) (34) or a marker for medium and large neurons (NF200) (35). The DRGs from sham and SNL rats were cut at 30 μm and collected free floating in 0.1 M PBS. Sections were rinsed in Tris-HCl buffer and incubated with 1% H₂O₂ in TBS for 30 min to quench the endogenous peroxidase. Sections were blocked with 5% blocking agent (PerkinElmer Life Sciences) in 0.1 M Tris-HCl for 1 h at 25 °C. Then the sections were incubated with the primary antibody mixture as follows: rabbit anti-Cav β3 (Alomone Labs, Jerusalem, Israel; dilution 1:100) and mouse anti-NF200 (Sigma; dilution 1:200) or mouse anti-peripherin (Abcam, Cambridge, MA; dilution 1:100) at 25 °C for 2 h and at 4 °C overnight. Subsequently, sections were rinsed and incubated with the secondary antibody mixture as follows: peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch; dilution 1:100) and Alexa Fluor-594-conjugated donkey anti-mouse IgG (Molecular Probes, Eugene, OR; dilution 1:400) for 2 h at room temperature. Then the sections were rinsed and incubated with fluorescein tyramide (PerkinElmer Life Sciences; dilution 1:100) for 10 min. Finally, the sections were rinsed, mounted on slides, dried, and coverslipped. The negative control was established by omitting the primary antibody.
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The sections were examined on a laser-scanning confocal microscope (Carl Zeiss, Jena, Germany), and the areas of interest were photo-documented. To quantify changes in the distribution of Cavβ3 in peripherin- and NF200-immunoreactive DRG neurons by nerve injury, four confocal images were randomly selected from each DRG (two DRGs/rat) in three control DRG neurons by nerve injury, four confocal images were randomly selected from each DRG (two DRGs/rat) in three control rats, as we described previously (36). Chitosan is a biodegradable cationic polysaccharide that binds tightly with the negatively charged siRNA to form the chitosan-siRNA nanoparticles. After recovery from catheter cannulation for 2 days, the specific siRNA to Cavβ3, Cavβ2, Cavβ4 subunits in the DRG and dorsal spinal cord tissues (NC) (n = 4 in each group). Ipsilateral (nerve injury) side; Cont, contralateral (control) side. *p < 0.05, compared with the corresponding value on the contralateral (control) side.

FIGURE 1. Effect of nerve injury on the mRNA level of Cavβ subunits in the DRG and dorsal spinal cord tissues. A, agarose gel electrophoresis showing the presence of the DNA products of Cavβ3, Cavβ2, and Cavβ4 subunits in the DRG and dorsal spinal cord (SC) tissues from control rats. G1A1 cells stably expressing Cavβ3 were used as a positive control for the Cavβ3 subunit, and the HEK293A cell line was used as a negative control. β group data show the effect of SNL on the mRNA level of Cavβ3, Cavβ2, and Cavβ4 subunits in the DRG (n = 4 in each group). C, summary data show the effect of SNL on the mRNA level of Cavβ3, Cavβ2, and Cavβ4 subunits in the dorsal spinal cord (SC) (n = 4 in each group). Ipsilateral (nerve injury) side; Cont, contralateral (control) side. *p < 0.05, compared with the corresponding value on the contralateral (control) side.

High Expression Levels of Cavβ3 and Cavβ4 Subunits in the DRG and Spinal Cord—To determine which Cavβ subunits are present in the DRG and dorsal spinal cord, we first used reverse transcriptase-PCR and agarose gel electrophoresis to detect the mRNA levels of Cavβ1, Cavβ2, Cavβ3, and Cavβ4 subunits in DRG and spinal cord tissues (Fig. 1A), but Cavβ1 mRNA was not detected in the DRG or spinal cord on the agarose gel. However, Cavβ1 (463 bp) was clearly present in G1A1 cells, a HEK293-derived cell line that stably expresses Cavβ1, Cavβ2, and Cavβ4 subunits (37).

We then quantified the mRNA level of individual Cavβ subunits in the DRG and dorsal spinal cord by using real time PCR. In the L5/L6 DRGs obtained from control rats, the Ct values of Cavβ1, Cavβ2, Cavβ3, and Cavβ4 subunits were 28.32, 24.92, 21.74, and 21.59, respectively. Thus, Cavβ3 and Cavβ4 were the most abundant Cavβ subtypes present in the DRG. We calculated (I-V) curves for individual neurons were constructed by calculating the peak inward current at each testing potential and normalizing to the cell capacitance. Conductance voltage (G-V) curves were fit with the Boltzmann equation as follows: G/G_max = 1/(1 + exp(Vm - V_0.5/k)), where V_0.5 is the voltage for 50% activation or inactivation of HVACCs, and k is a voltage-dependent slope factor. Results were expressed as mean ± S.E. Student’s t test was used to compare two groups, and one-way analysis of variance (with Tukey’s or Dunnett’s post hoc test) was used to compare multiple groups. p < 0.05 was considered statistically significant.

RESULTS

Data Analysis—The HVACC current data were analyzed by using the PulseFit software program (HEKA Instruments). The amplitude of monosynaptic EPSCs was analyzed by using Clampfit 9.2 (Molecular Devices). The whole-cell current voltage (I-V) curves for individual neurons were constructed by calculating the peak inward current at each testing potential and normalizing to the cell capacitance. Conductance voltage (G-V) curves were fit with the Boltzmann equation as follows: G/G_max = 1/(1 + exp(Vm - V_0.5/k)), where V_0.5 is the voltage for 50% activation or inactivation of HVACCs, and k is a voltage-dependent slope factor. Results were expressed as mean ± S.E. Student’s t test was used to compare two groups, and one-way analysis of variance (with Tukey’s or Dunnett’s post hoc test) was used to compare multiple groups. p < 0.05 was considered statistically significant.

Chitosan-siRNA Preparation and Intrathecal Injection—All of the siRNA was purchased from Integrated DNA Technologies (San Diego). Two Cavβ3-specific siRNAs (IDT catalog numbers 57372397 and 57372400) and a control siRNA (IDT catalog number 580088661; target sense, CGUUAACGCAGAUAAUACGCGUAT) were diluted by using RNA-free duplex buffer (1 μg/μl), incubated at 94 °C for 2 min for annealing, and then cooled slowly to room temperature (~25 °C). We used chitosan to conjugate the siRNA for intrathecal injections in rats as we described previously (36). Chitosan-siRNA Preparation and Intrathecal Injection—All of the siRNA was purchased from Integrated DNA Technologies (San Diego). Two Cavβ2-specific siRNAs (IDT catalog numbers 57372397 and 57372400) and a control siRNA (IDT catalog number 580088661; target sense, CGUUAACGCAGAUAAUACGCGUAT) were diluted by using RNA-free duplex buffer (1 μg/μl), incubated at 94 °C for 2 min for annealing, and then cooled slowly to room temperature (~25 °C). We used chitosan to conjugate the siRNA for intrathecal injections in rats as we described previously (36). Chitosan-siRNA Preparation and Intrathecal Injection—All of the siRNA was purchased from Integrated DNA Technologies (San Diego). Two Cavβ2-specific siRNAs (IDT catalog numbers 57372397 and 57372400) and a control siRNA (IDT catalog number 580088661; target sense, CGUUAACGCAGAUAAUACGCGUAT) were diluted by using RNA-free duplex buffer (1 μg/μl), incubated at 94 °C for 2 min for annealing, and then cooled slowly to room temperature (~25 °C). We used chitosan to conjugate the siRNA for intrathecal injections in rats as we described previously (36).
lulated the relative mRNA levels of Cavβ subunits by using the $2^{-\Delta\Delta Ct}$ method (the Ct value of GAPDH was 22.59 in the DRG). When we considered the mRNA level of Cavβ3 to be 100%, the relative mRNA levels of Cavβ1, Cavβ2, and Cavβ4 subtypes were 0.94, 9.94, and 90.13%, respectively, of Cavβ4 in the DRG tissue.

In the dorsal spinal cord obtained from control rats, the Ct values of the Cavβ1, Cavβ2, Cavβ3, and Cavβ4 were 29.88, 23.53, 23.18, and 21.19, respectively. The Ct value of GAPDH in the spinal cord was 22.61. When we considered the mRNA level of Cavβ3 to be 100%, the relative mRNA levels of Cavβ1, Cavβ2, and Cavβ3 subtypes were 0.48, 19.75, and 25.17%, respectively, of Cavβ4 in the spinal cord tissue. Because the expression level of Cavβ1 in the DRG and spinal cord is very low, we did not study the effect of nerve injury on Cavβ1 subunit expression in the following experiments.

**Nerve Injury Increases the Expression Level of the Cavβ3 but Not Cavβ4 Subunit in the DRG and Spinal Cord**—We next used quantitative PCR to determine how nerve injury changes the expression levels of Cavβ2, Cavβ3, and Cavβ4 subunits in the DRG and dorsal spinal cords contralateral and ipsilateral to SNL. All of the rats developed tactile allodynia and hyperalgesia within 7 days after SNL surgery. We analyzed changes in the mRNA and protein levels of Cavβ subunits in the DRG and dorsal spinal cord 9 days after SNL. The Cavβ3 mRNA level in the DRG ipsilateral to SNL was significantly reduced (23.13% of control level). In contrast, the mRNA level of the Cavβ3 subunit was about 1.57 times greater in the DRG ipsilateral to SNL than in the contralateral DRG. The Cavβ3 mRNA level in the DRG did not differ significantly between the ipsilateral and contralateral sides (Fig. 1B). In the dorsal spinal cord, the Cavβ2 mRNA level was 53.64% lower in the ipsilateral side than the contralateral side of SNL rats. The mRNA level of Cavβ4 and Cavβ3 in the dorsal spinal cord did not differ significantly between the ipsilateral and the contralateral sides (Fig. 1C).

Similarly, the Cavβ3 protein level in the DRG ipsilateral to SNL was about 1.9 times that of the contralateral side (Fig. 2A). However, the Cavβ3 protein level in the DRG did not differ significantly between the ipsilateral and contralateral sides (Fig. 2B). In the dorsal spinal cord, the protein level of Cavβ3 and Cavβ4 did not differ significantly between the ipsilateral and contralateral sides of SNL rats (Fig. 2A and B). Because the Cavβ3 protein band of Cavβ3 and Cavβ4 in the DRG and dorsal spinal cord was below the detection level in the immunoblots, we did not quantify nerve injury-induced changes in the protein level of these two subunits. Therefore, our findings indicate that peripheral nerve injury primarily increases the expression level of the Cavβ3 subunit in the DRG.

**Changes in Distribution of the Cavβ3 Subunit in DRG Neurons after Nerve Injury**—We then used double immunofluorescence labeling and confocal microscopy to examine changes in the distribution of the Cavβ3 subunit in NF200- and peripherin-immunoreactive DRG neurons in SNL rats. The Cavβ3 immunoreactivity was present in most DRG neurons immunoreactive to NF200 in control rats (511/547, 93.42%). However, only 44.82% (389/868) peripherin-immunoreactive DRG neurons are co-localized with the Cavβ3 subunit in sham control rats (Fig. 3). In DRG sections from nerve-injured rats, Cavβ3 immunoreactivity was present in 95.31% (447/469) NF200-immunoreactive neurons. Notably, the number of peripherin-immunoreactive DRG neurons co-localized with Cavβ3 (783/822, 95.26%) was significantly increased in nerve-injured rats compared with that in control rats (Fisher’s exact test, Fig. 3).

**Nerve Injury Increases HVACC Activity in Small DRG Neurons and Glutamatergic Input to Spinal Dorsal Horn Neurons**—Although complete nerve transection (axotomy) can reduce HVACC activity in DRG neurons (38), the effect of spinal nerve ligation injury on HVACC activity has not been specifically examined. To determine the changes in the HVACC activity in DRG neurons by SNL, we recorded and compared HVACC currents ($I_{Ba}$) in rat DRG neurons 9–10 days after SNL and sham surgery. Neurons were voltage-clamped at $-90$ mV and depolarized to 0 mV for 200 ms at 1-s intervals. In small (<30 μm) DRG neurons, the peak amplitude of $I_{Ba}$ was 1.6 times greater in SNL rats than in sham control rats (Fig. 4A). However, in medium (30–40 μm) and large (>40 μm) DRG neurons, there were no significant differences in the current density of $I_{Ba}$ between SNL and sham rats (Fig. 4A).

We also determined whether SNL increases glutamatergic input from primary afferent terminals to spinal dorsal horn neurons. EPSCs of lamina II neurons were evoked by electrical stimulation of the dorsal root. The EPSCs were considered monosynaptic if the latency was constant after electrical stimulation (0.2 Hz) and if no conduction failure or increased latency occurred when stimulation frequency was increased to 20 Hz (30, 32). The amplitude of monosynaptic EPSCs of lamina II neurons was significantly increased in the ipsilateral side compared with the contralateral side in SNL rats (Fig. 4B). Blocking glutamate AMPA receptors with 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione abolished EPSCs in all lamina II neurons tested (39).

Next, we determined whether SNL affects activation and inactivation kinetics of $I_{Ba}$ in DRG neurons. The whole-cell HVACC currents in DRG neurons were evoked by a series of depolarizing pulses (from $-70$ to 50 mV for 150 ms in 10-mV increments) from the holding potential of $-90$ mV. The cur-
rent density of $I_{\text{Ba}}$ in small DRG neurons was largely increased in SNL rats compared with that in sham control rats (Fig. 4C). Although SNL did not alter the current-voltage relationship of $I_{\text{Ba}}$, it caused a significant hyperpolarizing shift in $V_{0.5}$ in small DRG neurons (SNL, $-17.5 \pm 0.1$ mV; control, $-13.7 \pm 0.1$ mV) (Fig. 4D). However, SNL did not significantly alter the voltage-dependent inactivation of $I_{\text{Ba}}$ in DRG neurons ($n = 15–26$ in each group, see Fig. 4E).

**FIGURE 3.** Nerve injury induces increased Cavβ3 in peripherin-immunoreactive DRG neurons. Representative confocal images show the distribution of Cavβ3 immunoreactivity (green) in small and large DRG neurons obtained from a sham control and an SNL rat. Neurons that are immunoreactive to NF200 or peripherin are indicated in red. Scale bar, 50 μm. All images are single confocal optical sections.

Down-regulation of Cavβ3 Subunit Reduces HVACC Activity in DRG Neurons and Glutamatergic Input to Spinal Dorsal Horn Neurons—Small interfering RNA (siRNA) (20–30 nucleotides) can silence target genes by degrading mRNA with the matching sequence in vivo and in vitro. It has been shown that 27 nucleotides are more effective in suppressing a target gene than are 21 nucleotides (40). We thus designed two sequences of siRNA with 27 nucleotides targeting the Cavβ3 subunit. To ensure the efficient delivery of siRNA into the spinal cord and DRG neurons, we incorporated the siRNA into chitosan (36). In our preliminary experiments, intrathecal treatment with the first Cavβ3-siRNA (5’-AUGUCUCUCCUCAUGCUACAUUGCCU-3’) reduced the Cavβ3 protein level in the DRG by ~50%. However, the second Cavβ3-siRNA (5’-AUCCACCA-GUCAUUGCUGUACUUCU-3’) decreased the Cavβ3 protein level by only 20%. Because the first Cavβ3 siRNA had a much better effect than did the second Cavβ3 siRNA, we used the first Cavβ3-specific siRNA in the following experiments.

We injected siRNA (5 μg) via intrathecal catheters starting on day 10 after SNL for 3 consecutive days.
level in the ipsilateral side and a 41.75% reduction in the contralateral side of SNL rats, compared with that in control siRNA-treated rats (Fig. 5A). In the dorsal spinal cord, intrathecal treatment with Cavβ3-specific siRNA decreased the Cavβ3 mRNA level by 40% in both sides, compared with that in control siRNA-treated rats (Fig. 5B). To ensure the specificity of the Cavβ3 siRNA, we also determined the effect of Cavβ3-specific siRNA on the Cavβ3 mRNA level in the DRG and dorsal spinal cord. The Cavβ3 siRNA had no significant effects on the Cavβ3 mRNA level in the DRG and spinal cord of SNL rats (Fig. 5, A and B).

Treatment with the Cavβ3-specific siRNA caused a larger reduction in the Cavβ3 protein level in the DRG on the ipsilateral side and a 41.75% reduction in the contralateral side of SNL rats, compared with that in control siRNA-treated rats (Fig. 5A).

**FIGURE 4.** Effects of nerve injury on HVACC activity of DRG neurons and monosynaptic EPSCs of spinal dorsal horn neurons. **A,** original current traces (upper panel) show HVACC currents in small DRG neurons from a control and a nerve-injured rat. Group data (lower panel) show that SNL increased HVACC currents in small, but not large and medium, DRG neurons. Neurons were voltage-clamped at −90 mV and depolarized to 0 mV for 200 ms. The number of cells in each group is indicated in the column. **B,** representative traces and group data show the monosynaptic EPSCs of lamina II neurons in spinal cords obtained from sham control (n = 19) and SNL (n = 20) rats. **C,** effect of SNL on the current-voltage relationship of HVACCs in small, medium, and large DRG neurons. **D,** effect of SNL on voltage-dependent activation of HVACCs in small, medium, and large DRG neurons. Note that SNL shifted voltage-dependent activation of HVACCs to the left only in small DRG neurons. The $V_{0.5}$ in control and SNL rats was $−13.7 ± 0.1$ and $−17.5 ± 0.1$ mV ($p < 0.05$), respectively. The slope factor in control and SNL rats was $8.33 ± 0.2$ and $7.80 ± 0.1$ mV ($p < 0.05$), respectively. **E,** effect of SNL on voltage-dependent inactivation of HVACCs in small, medium, and large DRG neurons. *, $p < 0.05$, compared with the corresponding value in the sham group. pF, picofarad.
eral side (60.90% decrease) than on the contralateral side (40.92% decrease) (Fig. 5C). Also, the amount of the Cavβ3/β4 protein on both sides of the dorsal spinal cord was significantly reduced (by ~40%) in rats treated with the Cavβ3-specific siRNA, compared with that in control siRNA-treated rats (Fig. 5D).

To determine the contribution of the Cavβ3 subunit to increased HVACC activity in DRG neurons of SNL rats, we examined the effect of Cavβ3-specific siRNA on the current density of $I_{Ba}$ in small DRG neurons ipsilateral to SNL, compared with the control siRNA-treated rats (Fig. 6A and C). By comparison, treatment with Cavβ3-siRNA caused a smaller reduction in the current density of $I_{Ba}$ in small DRG neurons on the contralateral side, compared with the control siRNA-treated rats. Also, treatment with Cavβ3-siRNA caused a depolarizing shift of $V_{0.5}$ in small DRG neurons ipsilateral to SNL (control siRNA, $-14.37 \pm 0.1$; Cavβ3-specific siRNA, $-11.02 \pm 0.1$ mV; see Fig. 6D).

Intrathecal treatment with Cavβ3-specific siRNA also significantly decreased the amplitude of monosynaptic EPSCs of spinal lamina II neurons evoked from the dorsal root in SNL rats (Fig. 6B). This Cavβ3 siRNA effect was much more prominent in the ipsilateral than in the contralateral side of the spinal cord in SNL rats. Collectively, these data indicate that up-regulation of the Cavβ3 subunit in primary afferent neurons contributes to increased activity of HVACCs and glutamatergic input from primary afferents to spinal dorsal horn neurons caused by nerve injury.

**Down-regulation of Cavβ3 Subunit at the Spinal Level Reduces Neuropathic Pain**—In addition, we performed nociceptive behavioral tests to determine whether up-regulation of the Cavβ3 subunit in DRG neurons contributes to pain hypersensitivity induced by nerve injury. SNL significantly reduced the pressure and tactile withdrawal thresholds of the hind paw on the ipsilateral side compared with the contralateral side (Fig. 7). Intrathecal treatment with Cavβ3-specific siRNA (5 μg/day for 3 days) or control siRNA had no significant effect on the ambulation behavior of rats. Compared with the control siRNA...
treatment, intrathecal administration of Cavβ3-specific siRNA gradually reversed the pressure and tactile withdrawal thresholds of the hind paw ipsilateral to SNL (Fig. 7). The pressure withdrawal threshold and tactile withdrawal threshold of the hind paw ipsilateral to SNL were completely normalized 3 days after treatment with Cavβ3-specific siRNA. However, treatment with control siRNA did not significantly alter the pressure and tactile withdrawal thresholds of the hind paw ipsilateral to SNL. Also, the Cavβ3-specific siRNA had no significant effect on the pressure and tactile withdrawal thresholds of the hind paw contralateral to SNL (Fig. 7).

**DISCUSSION**

In this study, we determined how nerve injury affects the expression of Cavβ subunits in primary sensory neurons and whether such changes contribute to increased HVACC cur-
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The pore-forming α₁ subunit is often associated with two core auxiliary subunits consisting of the membrane-anchored, largely extracellular α₂δ subunit and the cytoplasmic Cavβ subunit (5). The α₁ subunits are the primary determinants of both channel biophysics and pharmacology, whereas Cavβ, α₂δ, and γ subunits are regarded as regulators of HVACC functions. There are four subfamilies of Cavβ subunits that are mainly expressed in the excitable tissues such as the brain, heart, and muscles. Cavβ₁ is mainly expressed in the hippocampus and skeletal muscle (46), whereas Cavβ₂ is mostly expressed in the heart (47, 48). Cavβ₃ is expressed not only in several brain regions but also in the heart and lungs (47, 49). Cavβ₄ is strongly expressed in the cerebellum (14). It has been reported that the mRNA level of several α₁ subunits in DRG tissues is reduced by sciatic nerve injury (50). Although nerve injury can increase α₂δ expression in DRG neurons (51, 52), it is uncertain whether this change is casually related to increased HVACC activity in DRG neurons by nerve injury. α₂B (N-type) is the predominant α₁ subtype that mediates HVACC currents in DRG neurons (25). The β₂ subunit regulates both L- and N-type HVACCs (53, 54). By using quantitative PCR and Western blot analyses, we found that Cavβ₂ and Cavβ₃ are the most predominant Cavβ subtypes distributed in the DRG and dorsal spinal cord tissues. Furthermore, we found that SNL caused a significant increase in the mRNA and protein levels of the Cavβ₂, but not Cavβ₃ subunit in the DRG, suggesting that the Cavβ₂ expression level is increased in primary afferent neurons in neuropathic pain. Our immunolabeling results provide further evidence that increased Cavβ₂ expression occurs predominantly in peripherin-positive small-sized DRG neurons after nerve injury. We noted that nerve injury significantly decreased the mRNA level of Cavβ₂ in the DRG and spinal cord. Because the basal expression level of Cavβ₂ in the DRG and spinal cord was extremely low, the reduced Cavβ₂ expression level may have little impact on HVACC activity in primary sensory neurons. Alternatively, the reduced Cavβ₂ level may free up HVACC α₁ subunits and allow them to form more α₁-Cavβ₂ complexes in injured DRG neurons.

Although the important role of the Cavβ₂ subunit in the regulation of HVACCs has been shown in cell lines (12, 26), their contribution to increased HVACC activity in neuropathic pain has not been demonstrated in native neurons due to the lack of specific pharmacological approaches. In this study, we demonstrated that intrathecal treatment with Cavβ₂-specific siRNA conjugated to chitosan selectively and efficiently knocked down the expression level of the Cavβ₂ subunit in the DRG and dorsal spinal cord. Importantly, down-regulation of Cavβ₂ by siRNA caused a large reduction in the current density of HVACCs and a depolarizing shift in the voltage-dependent activation of HVACCs in small DRG neurons ipsilateral to SNL. By comparison, Cavβ₁-specific siRNA had little effect on HVACC currents in small DRG neurons on the control side of SNL rats. Cavβ₁-specific siRNA also reduced voltage-dependent activation and inactivation of HVACC currents in small DRG neurons, which is consistent with the role of Cavβ₁ in the control of HVACC gating (15). Thus, our results indicate that up-regulation of the β₂ subunit plays a key role in increased HVACC activity in injured DRG neurons in neuropathic pain. Because treatment with Cavβ₂-specific siRNA attenuated the amplitude
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of monosynaptic EPSCs of dorsal horn neurons more on the ipsilateral side than on the contralateral side of SNL rats, this finding provides further evidence that up-regulation of the Cavβ3 subunit contributes to increased HVACC activity and neurotransmitter release from the primary afferent terminals in the spinal dorsal horn in neuropathic pain. An increase in the Cavβ3 subunit could increase the α,β-Cavbinding and augment HVACC activity by promoting α2 subunit expression on the plasma membrane after nerve injury.

Another salient finding of our study is that down-regulation of the Cavβ3 subunit at the spinal level by chitosan-siRNA profoundly reduced mechanical hyperalgesia and tactile allodynia induced by SNL in rats. We found that intrathecal treatment with Cavβ3-specific siRNA for 3 days completely normalized pain hypersensitivity in SNL rats. Interestingly, although knockdown of Cavβ3 also decreased the expression level of Cavβ3 in DRG neurons and spinal cord contralateral to SNL, intrathecal treatment with Cavβ3-specific siRNA had no significant effect on the nociceptive and tactile thresholds of the hind paw contralateral to SNL. This finding suggests that down-regulation of Cavβ3 at the spinal level alone does not impair normal nociception. Consistent with our finding with the siRNA approach, it has been reported that knock-out of Cavβ3 in mice reduces acute inflammatory pain but has little effect on normal nociceptive thresholds in response to noxious heat and mechanical stimuli (55). The profound effect of Cavβ3-specific siRNA on SNL-induced pain hypersensitivity suggests that the increased Cavβ3 expression level in DRG neurons becomes critically important for augmented HVACC activity and nociceptive input in neuropathic pain. This notion is supported by our finding that the Cavβ3-siRNA effect on the HVACC currents of small DRG neurons was much more pronounced on the ipsilateral (injury) side than the contralateral (control side) of SNL rats. Furthermore, the inhibitory effect of Cavβ3-specific siRNA on the evoked EPSC amplitude of spinal dorsal horn neurons was much greater on the ipsilateral side than on the contralateral side of SNL rats. Another possibility is that nerve injury may increase the uptake of chitosan-siRNA by injured primary afferent neurons. This is because Cavβ3-specific siRNA treatment produced a greater effect on the Cavβ3 expression level in the DRG on the ipsilateral side than on the contralateral side of SNL rats.

In summary, we demonstrated in this study that the Cavβ3 subunit is up-regulated and critically contributes to increased HVACC activity in DRG neurons and nociceptive input to spinal dorsal horn neurons in neuropathic pain. Down-regulation of the Cavβ3 subunit at the spinal level normalizes neuropathic pain but has no significant effect on normal nociception. Directly blocking N-type HVACCs at the spinal level often produces intolerable adverse effects (42, 56) and thus has limited clinical use in the treatment of chronic pain. Our findings suggest the Cavβ3 subunit may represent a new target for neuropathic pain treatment with a reduced adverse effect profile. Many aminopyridine analogs can modulate HVACC activity through the Cavβ subunit (26). These compounds could be modified to inhibit Cavβ3 subunit function to reduce increased HVACC activity and nociceptive input in neuropathic pain. Therefore, our study provides important information about the molecular mechanisms underlying increased HVACC activity in primary sensory neurons by nerve injury. This new information improves our understanding of neuropathic changes associated with neuropathic pain and helps the design of novel analgesics for neuropathic pain treatment.

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