

Casein Kinase II Regulates *N*-Methyl-D-Aspartate Receptor Activity in Spinal Cords and Pain Hypersensitivity Induced by Nerve Injury

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ABSTRACT

Increased *N*-methyl-D-aspartate receptor (NMDAR) activity and phosphorylation in the spinal cord are critically involved in the synaptic plasticity and central sensitization associated with neuropathic pain. However, the mechanisms underlying increased NMDAR activity in neuropathic pain conditions remain poorly understood. Here we show that peripheral nerve injury induces a large GluN2A-mediated increase in NMDAR activity in spinal lamina II, but not lamina I, neurons. However, NMDAR currents in spinal dorsal horn neurons are not significantly altered in rat models of diabetic neuropathic pain and resiniferatoxin-induced painful neuropathy (postherpetic neuralgia). Inhibition of protein tyrosine kinases or protein kinase C has little effect on NMDAR currents potentiated by nerve injury. Strikingly, casein kinase II (CK2) inhibitors normalize increased NMDAR currents of dorsal

horn neurons in nerve-injured rats. In addition, inhibition of calcineurin, but not protein phosphatase 1/2A, augments NMDAR currents only in control rats. CK2 inhibition blocks the increase in spinal NMDAR activity by the calcineurin inhibitor in control rats. Furthermore, nerve injury significantly increases CK2 α and CK2 β protein levels in the spinal cord. In addition, inhibition of CK2 or CK2 β knockdown at the spinal level substantially reverses pain hypersensitivity induced by nerve injury. Our study indicates that neuropathic pain conditions with different etiologies do not share the same mechanisms, and increased spinal NMDAR activity is distinctly associated with traumatic nerve injury. CK2 plays a prominent role in the potentiation of NMDAR activity in the spinal dorsal horn and may represent a new target for treatments of chronic pain caused by nerve injury.

Introduction

Chronic neuropathic pain, typically caused by damage to or a dysfunction of the peripheral or central nervous system, remains a major clinical problem and therapeutic challenge. Existing analgesics for neuropathic pain are not very effective and are associated with various side effects. One of the major obstacles in developing new therapies for neuropathic pain is that we have limited understanding of the mechanisms underlying the transition from acute to chronic pain after nerve injury. Both peripheral sensitization at the injury site and hyperactivity of the spinal dorsal horn neurons result in pain hypersensitivity after nerve injury (Campbell et al., 1988; Gracely et al., 1992). Increased glutamatergic synaptic input to spinal dorsal horn neurons plays a critical role in the

development of central sensitization and neuropathic pain (Chaplan et al., 1997; Wang et al., 2007; Zhou et al., 2011a; Li et al., 2012b). In addition, increased *N*-methyl-D-aspartate receptor (NMDAR) activity by nerve injury diminishes normal synaptic inhibition mediated by GABA and glycine in the spinal cord through increased proteolysis of K⁺-Cl⁻ cotransporter-2 mediated by calpain (Zhou et al., 2012).

Because the NMDAR is critically involved in synaptic plasticity and the development of neuropathic pain after peripheral nerve injury, determining the molecular mechanisms underlying potentiated NMDAR activity in neuropathic pain is particularly important. Increased NMDAR activity in the spinal cord after nerve injury likely results from increased phosphorylation of NMDARs (Gao et al., 2005; Zhou et al., 2012). However, the specific protein kinases responsible for increased NMDAR activity caused by nerve injury have not been conclusively identified. Several protein kinases, including protein kinase C (PKC) (Chen and Huang, 1992), protein kinase A (Raman et al., 1996), Ca²⁺/calmodulin-dependent protein kinase II (Kolaj et al., 1994), and protein tyrosine kinase (Yu et al., 1997), can modulate NMDAR function.

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ABBREVIATIONS: aCSF, artificial cerebrospinal fluid; AMPAR, α -amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid receptor; AP5, *dl*-2-amino-5-phosphonovaleric acid; CK2, casein kinase II; DRB, 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside; EPSC, excitatory postsynaptic current; mEPSC, miniature excitatory postsynaptic current; NMDAR, *N*-methyl-D-aspartate receptor; PCR, polymerase chain reaction; PKC, protein kinase C; PP2, 3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine; Ro 25-6981, (α R, β S)- α -(4-hydroxyphenyl)- β -methyl-4-(phenylmethyl)-1-piperidinepropanol maleate; RTX, resiniferatoxin; siRNA, small interfering RNA; SNL, spinal nerve ligation; STZ, streptozotocin; TBB, 4,5,6,7-tetrabromobenzotriazole.

Although inhibition of spinal PKC and protein tyrosine kinase Src reduces pain hypersensitivity induced by nerve injury (Hua et al., 1999; Katsura et al., 2006), there is no direct evidence linking these kinases to increased NMDAR activity of spinal dorsal horn neurons in neuropathic pain.

Furthermore, treatment with NMDAR antagonists can lead to long-lasting relief for patients with nerve injury-induced neuropathic pain (Rabben et al., 1999; Correll et al., 2004). By contrast, clinical studies indicate that NMDAR antagonists have no or limited effects on neuropathic pain caused by diabetic neuropathy and postherpetic neuralgia (Nelson et al., 1997; Sang et al., 2002). It is not clear why NMDAR antagonists have such inconsistent analgesic effects in different neuropathic pain conditions. This study is intended to fill these knowledge gaps by directly determining changes in the NMDAR activity of dorsal horn neurons in different neuropathic pain conditions and by identifying specific protein kinases that are critically involved in the potentiation of spinal NMDAR activity in neuropathic pain.

Here we demonstrate that spinal NMDAR activity is profoundly increased after peripheral nerve injury but not in painful diabetic neuropathy and resiniferatoxin (RTX)-induced painful neuropathy (a rat model of postherpetic neuralgia). The effectiveness of NMDAR antagonists in treating neuropathic pain is still controversial, and our study provides new evidence that warrants the revisiting of this class of drugs for the treatment of different neuropathic pain conditions. We also show that increased casein kinase II (CK2) activity is largely responsible for the enhanced NMDAR-mediated synaptic transmission in the spinal dorsal horn and the pain hypersensitivity caused by nerve injury.

Materials and Methods

Rat Model of Peripheral Nerve Injury. A total of 236 male Sprague-Dawley rats (aged 8 weeks; Harlan, Indianapolis, IN) were used for the entire study. All of the surgical preparation and experimental protocols were approved by the University of Texas MD Anderson Cancer Center Animal Care and Use Committee and conformed to the National Institutes of Health guidelines for the ethical use of animals. Spinal nerve ligation (SNL) was used as an experimental model of neuropathic pain in our study. We induced anesthesia with 2–3% isoflurane and then isolated the left L5 and L6 spinal nerves and ligated them with a 5-0 silk suture. Control animals underwent a sham surgical procedure without nerve ligation. In this SNL model, stable pain hypersensitivity is typically established 10–14 days after SNL and lasts for at least 8 weeks. Final electrophysiological recordings were done 3 weeks after surgery except the time course experiments.

Intrathecal catheters were implanted in some SNL and sham-operated rats during isoflurane-induced anesthesia 1 week after surgery. Briefly, we made a small incision at the back of the animal's neck. Next, we made a small opening in the atlanto-occipital membrane of the cisterna magna and inserted a PE-10 catheter (approximately 8.0 cm) such that the caudal tip reached the lumbar spinal cord (Chen and Pan, 2005). The rostral end of the catheter was exteriorized, and the wound was closed with sutures. The animals were allowed to recover for 4 to 5 days after the surgery. Animals displaying signs of motor or neurologic dysfunction were excluded from the study.

Rat Model of Postherpetic Neuralgia Induced by RTX. We previously showed that a single systemic treatment with RTX, an ultrapotent transient receptor potential vanilloid 1 agonist, in adult rats ablates transient receptor potential vanilloid 1-expressing dorsal root ganglion neurons and induces pathology (primarily damaging

small sensory neurons and afferent nerves) and symptoms that resemble postherpetic neuralgia, such as diminished thermal sensation and tactile allodynia (Pan et al., 2003; Chen and Pan, 2005). Rats received a single intraperitoneal injection of RTX (200 $\mu\text{g}/\text{kg}$; LC Laboratories, Woburn, MA) while under 2–3% isoflurane anesthesia, and the anesthesia was maintained for approximately 3 hours after RTX injection. RTX was dissolved in a mixture of 10% Tween-80 and 10% ethanol in normal saline. Rats in the control group received injection of vehicle alone. The final electrophysiological experiments were conducted 4 weeks after the RTX and vehicle injections, and tactile allodynia and impaired thermal withdrawal responses were confirmed in all RTX-treated rats, as previously described (Pan et al., 2003; Chen and Pan, 2005).

Rat Model of Painful Diabetic Neuropathy. For the induction of diabetic neuropathic pain, adult rats received a single intraperitoneal injection of streptozotocin (STZ; 50 mg/kg; Sigma-Aldrich, St. Louis, MO) freshly dissolved in 0.9% sterile saline (Chen and Pan, 2002). Diabetes (hyperglycemia) was confirmed in STZ-injected rats by measuring plasma glucose concentrations in blood samples from the tail vein using ACCU-CHEK test strips (Roche Diagnostics, Indianapolis, IN). After STZ injection, diabetic rats gradually developed allodynia and mechanical hyperalgesia within 3 weeks (Chen and Pan, 2002). Final electrophysiological experiments were performed on rats 3 to 4 weeks after STZ injection, and mechanical hyperalgesia was confirmed in all diabetic rats used for this study.

Behavioral Assessment of Tactile Allodynia. Rats were individually placed in suspended chambers on a mesh floor. After a 30-minute acclimation period, a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL) was applied perpendicularly to the plantar surface of the left hindpaw with sufficient force to bend the filament for 6 seconds. Brisk withdrawal or paw flinching was considered a positive response. In the absence of a response, the filament of the next greater force was applied. After a response, the filament of the next lower force was applied. The tactile stimulus producing a 50% likelihood of withdrawal response was calculated by using the “up-down” method (Chaplan et al., 1997).

Behavioral Assessment of Mechanical Hyperalgesia. Mechanical nociception of the hindpaw was measured by using the Ugo Basile Analgesimeter (Varese, Italy) (Chen and Pan, 2002). The device was activated by pressing a foot pedal, which activated a motor that applied a constant, increasing force on a linear scale. When the animal displayed pain by either withdrawing of the paw or vocalization, the pedal was immediately released and the animal's nociceptive threshold was read on the scale. A maximum of 400 g was used as a cutoff to avoid potential tissue injury to the animals. Nociceptive tests were conducted in rats 3 to 4 weeks after surgery.

Spinal Cord Slice Preparation and Electrophysiological Recordings. We removed the lumbar segment of the spinal cord by means of laminectomy during isoflurane-induced anesthesia. The spinal cord was immediately placed in an ice-cold sucrose artificial cerebrospinal fluid (aCSF) presaturated with 95% O₂ and 5% CO₂. The sucrose aCSF contained the following (in mM): 234 sucrose, 3.6 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 12.0 glucose, and 25.0 NaHCO₃. The spinal cord tissue was then placed in a shallow groove formed in a gelatin block and glued on the stage of a vibratome. Transverse slices (400 μm) of the spinal cord at the L5 to L6 level were cut in ice-cold sucrose aCSF and preincubated in Krebs solution oxygenated with 95% O₂ and 5% CO₂ at 34°C for at least 1 hour before being transferred to the recording chamber. The Krebs solution contained the following (in mM): 117.0 NaCl, 3.6 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 11.0 glucose, and 25.0 NaHCO₃. The spinal cord slice was placed in a glass-bottomed chamber (Warner Instruments, Hamden, CT) and continuously perfused with Krebs solution at 5.0 ml/min at 34°C maintained by an inline solution heater and a temperature controller.

The spinal lamina II, a translucent region in the superficial dorsal horn, was identified on an upright fixed-stage microscope with differential interference contrast/infrared illumination. Lamina II outer

TABLE 1

Sequences of two CK2 β -specific siRNAs and one control siRNA

These siRNAs were conjugated to chitosan and then administered through intrathecal catheters in rats.

Gene	Target Sequences	Position of Coding Sequence
Rat CK2 (NM_001035238)	CK2 β -specific siRNA 1: 5'-CAACCAGAGTGACTTGATT-3'	275–294
	CK2 β -specific siRNA 2: 5'-CAAGGAGACTTTGGCTACT-3'	383–402
	Scrambled control siRNA: 5'-AATTCTCCGAACGTGTACAGT-3'	

zone neurons (and in some experiments, large-diameter lamina I neurons) were selected for whole-cell patch-clamp recordings, as previously described (Zhou et al., 2010, 2012). We used a glass pipette (5–10 M Ω) filled with internal solution containing the following (in mM): 135.0 potassium gluconate, 5.0 TEA, 2.0 MgCl₂, 0.5 CaCl₂, 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, 0.5 Na-GTP, and 10 lidocaine *N*-ethyl bromide (adjusted to pH 7.2–7.4 with 1 M KOH; 290–300 mOsm). The input resistance was monitored, and the recording was abandoned if it changed more than 15%. All signals were recorded using an amplifier (MultiClamp700B; Axon Instruments Inc., Union City, CA), filtered at 1 to 2 kHz, digitized at 10 kHz, and stored for off-line analysis. We used electrical stimulation (0.2 milliseconds, 0.6 mA, and 0.1 Hz) of the dorsal root to evoke monosynaptic excitatory postsynaptic currents (EPSCs) (Zhou et al., 2008, 2010; Zhao et al., 2012). The AMPAR [α -amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptor]-EPSCs were recorded at a holding potential of –60 mV in the presence of 10 μ M bicuculline and 1 μ M strychnine. NMDAR-EPSCs were recorded at +40 mV in the presence of 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione, 10 μ M bicuculline, and 1 μ M strychnine. To specifically measure synaptic NMDAR activity, NMDAR- and AMPAR-mediated mixed miniature EPSCs (mEPSCs) were recorded at a holding potential of –60 mV in the presence of tetrodotoxin (1 μ M), bicuculline (10 μ M), and strychnine (1 μ M) in the absence of Mg²⁺. The AMPAR-mediated mEPSCs were isolated by adding the NMDAR antagonist AP5 (*dl*-2-amino-5-phosphonovaleric acid; 50 μ M). The average NMDAR-mediated mEPSCs were obtained after subtracting the average AMPAR-mediated mEPSCs from the average NMDAR- and AMPAR-mediated mixed mEPSCs. Because the NMDAR component of mEPSCs has a small amplitude and slow decay kinetic (Kimura and Matsuki, 2008), using the amplitude of NMDAR-mEPSCs would not be accurate to reflect the synaptic NMDAR activity. For this reason, we used the area under the curve to compare the changes in NMDAR-mediated mEPSCs. In some neurons, NMDAR currents were elicited by puff application of 100 μ M NMDA (Zhao et al., 2012) to the recorded neuron using a positive pressure system (4 psi, 15 milliseconds; Toohy Company, Fairfield, NJ). The tip of the puff pipette was placed 150 μ m away from the recorded neuron, and the recordings were performed using the extracellular solution containing 0.1 mM Mg²⁺, 10 μ M glycine, and 1 μ M tetrodotoxin at a holding potential of –60 mV and using the pipette internal solution containing the following (in mM): 110.0 Cs₂SO₄, 2.0 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, 10.0 HEPES, 2.0 MgATP, and 0.3 Na₂GTP (pH was adjusted to 7.25 with 1.0 M CsOH; 280–300 mOsm). For each protocol, four to six rats were used (and three to four neurons were generally recorded from each rat).

DRB (5,6-dichlorobenzimidazole 1- β -D-ribofuranoside) was purchased from Sigma-Aldrich. Chelerythrine, TBB (4,5,6,7-tetrabromobenzotriazole), PP2 [3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine], okadaic acid, and FK-506 (tacrolimus) were obtained from Tocris Bioscience (Ellisville, MO).

Small Interfering RNA–Chitosan Nanoparticles. To knock down CK2 β expression in the rat spinal cord, we selected two specific high-performance liquid chromatography-purified CK2 β -small interfering RNAs (siRNAs) (Sigma-Aldrich). The sequences for the CK2 β siRNA and control scrambled siRNA used in this study are listed in Table 1. Chitosan nanoparticles are an efficient means to deliver siRNA to the spinal cord neurons. Chitosan-siRNA nanoparticles were prepared as previously described (Cai et al., 2009; Zhou et al., 2012). All siRNAs were given intrathecally in rats 3 to 4 weeks after SNL.

Measurement of CK2 Subunit mRNA Levels. Three days after the last siRNA injection, animals were anesthetized with sodium pentobarbital (60 mg/kg i.p.). The rats were then decapitated, and the L5 to L6 dorsal spinal quadrants were collected. Total RNA was isolated using TRIzol (Life Technologies, Grand Island, NY) and were reverse transcribed. The cDNA was subjected to polymerase chain reaction (PCR) amplification to detect β -actin (forward: 5'-TGAACCTAAGGCCAACCGTGAAAAGAT-3'; reverse: 5'-GACCA-GAGGCATACAGGGACAACACAGC-3'), CK2 α (forward: 5'-CCAGCAC-CTTGTCTAGCCCCG-3'; reverse: 5'-CCTGCCATGCCAGCCGAAT-3'), and CK2 β (forward: 5'-GGCACCACCACACGGATGGC-3'; reverse: 5'-TTGCTGGCGCTTGGAGCTG-3'). Real-time PCR was performed

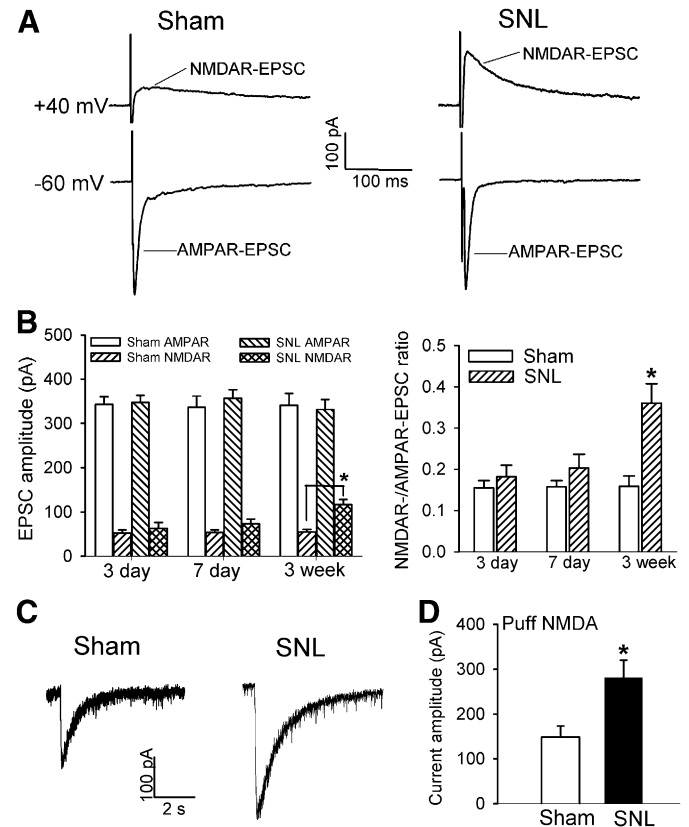


Fig. 1. Nerve injury potentiates NMDAR currents of spinal lamina II neurons. (A) Original recordings of evoked NMDAR-EPSCs and AMPAR-EPSCs of lamina II neurons recorded from one sham control and one SNL rat 3 weeks after surgery. The holding potential for recording NMDAR-EPSCs and AMPAR-EPSCs is indicated on the left. (B) Time course of mean changes in the amplitude and ratio of NMDAR-EPSCs to AMPAR-EPSCs of lamina II neurons recorded from sham control and SNL rats 3 days (10 and 14 neurons, respectively), 7 days (10 and 12 neurons, respectively), and 3 weeks (13 and 16 neurons, respectively) after surgery. (C and D) Representative traces (C) and mean changes (D) in the amplitude of NMDAR currents elicited by puff application of 100 μ M NMDA to lamina II neurons in sham control ($n = 16$ neurons) and SNL ($n = 19$ neurons) rats 3 weeks after surgery. * $P < 0.05$ compared with the control group. Error bars represent the S.E.M.

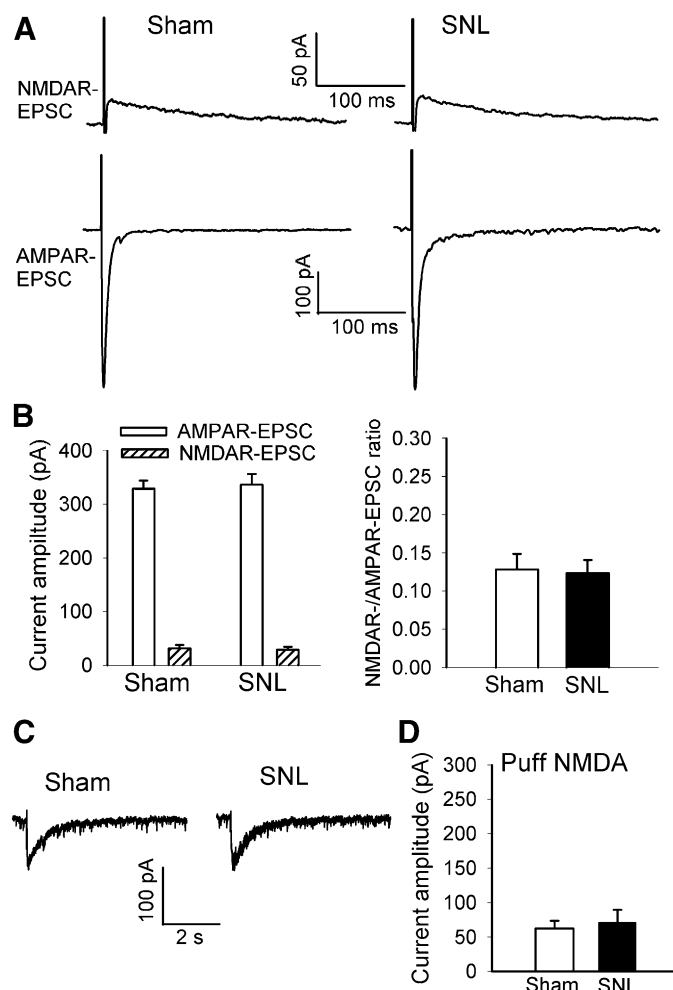


Fig. 2. Nerve injury has no effect on the NMDAR activity of lamina I neurons. (A and B) Original recordings (A) and mean changes (B) in the amplitude of NMDAR-EPSCs and AMPAR-EPSCs of lamina I neurons evoked by dorsal root stimulation in sham control ($n = 19$ neurons) and SNL ($n = 18$ neurons) rats 3 weeks after surgery. (C and D) representative traces (C) and mean changes (D) in the amplitude of NMDAR currents elicited by puff application of $100 \mu\text{M}$ NMDA to lamina I neurons in sham control ($n = 19$ neurons) and SNL ($n = 17$ neurons) rats. Error bars represent the S.E.M.

using the iQ5 real-time PCR system with the SYBR Green PCR core reagents kit (Bio-Rad Laboratories Inc., Hercules, CA). All samples were run in triplicate using a 60°C annealing temperature. For each sample, the relative amount of the target mRNA was first normalized to β -actin mRNA and then normalized by setting the mRNA level of the control siRNA-treated rats as 1 (Cai et al., 2009). The specificity of the PCR products was confirmed by melting curve analysis and agarose gel electrophoresis.

Western Blot Analysis of CK2 Proteins. To quantify the CK2 protein levels in the spinal cord after SNL, we obtained dorsal spinal cords at the L5 and L6 level from the left (SNL) and the contralateral (sham-operated) sides after anesthetizing rats with isoflurane. The tissues were homogenized in ice-cold buffer containing 20 mM Tris (pH 7.6), 0.5% NP40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 10% sucrose, and a protease inhibitor cocktail. The homogenate was then centrifuged at $12,000g$ for 20 minutes at 4°C . The supernatant was collected and the protein concentration was determined using the Lowry protein assay. For Western blotting, $50 \mu\text{g}$ protein was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 30 minutes in 5% skim milk in phosphate-buffered saline

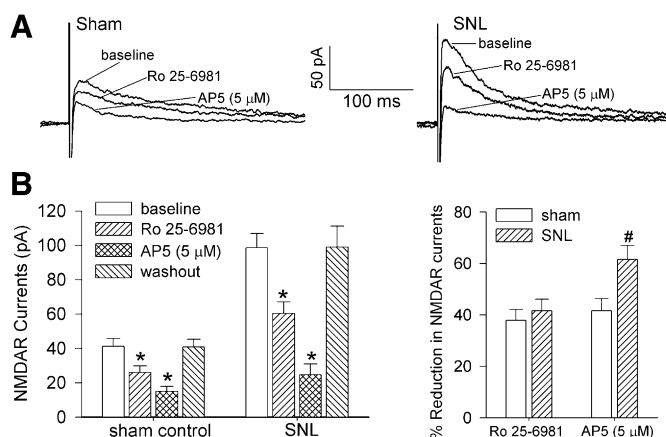


Fig. 3. GluN2A predominantly mediates the increased NMDAR activity of spinal lamina II neurons by nerve injury. (A) Representative recording traces show changes in evoked NMDAR-EPSCs of lamina II neurons during baseline control, bath application of $0.6 \mu\text{M}$ Ro 25-6981, and bath application of $5 \mu\text{M}$ AP5 plus $0.6 \mu\text{M}$ Ro 25-6981 from one sham rat and one SNL rat. (B) Mean changes in the amplitude of NMDAR currents of lamina II neurons during baseline control, bath application of $0.6 \mu\text{M}$ Ro 25-6981, bath application of $5 \mu\text{M}$ AP5 plus $0.6 \mu\text{M}$ Ro 25-6981, and washout in sham ($n = 11$ neurons) and SNL ($n = 12$ neurons) rats 3 weeks after surgery. * $P < 0.05$ compared with the respective baseline control; # $P < 0.05$ compared with the corresponding value in the sham group. Error bars represent the S.E.M.

containing 0.05% Tween-20 and then incubated with goat anti-CK2 α (sc-6476; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-CK2 β primary antibody (sc-46666; Santa Cruz Biotechnology) (Ye et al., 2012) overnight at 4°C . The membrane was then rinsed and incubated with horseradish peroxidase-conjugated anti-goat or anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) at 1:10,000 dilution for 1 hour at 26°C . The membrane was developed with an enhanced chemiluminescence kit (GE Healthcare, Pittsburgh, PA). For the protein loading control, membranes were probed with a rabbit anti- β -actin antibody (Sigma-Aldrich). The intensity of protein bands was captured digitally and analyzed quantitatively with AIS software (Imaging Research Inc., St. Catharines, ON, Canada).

Statistical Analysis. Data are presented as means \pm S.E.M. The amplitude of EPSCs and NMDAR currents was analyzed with Clampfit 9.2 (Axon Instruments, Sunnyvale, CA). For all of the electrophysiological and behavioral data, the unpaired t test was used to compare two groups and one-way analysis of variance (with Dunnett's or Tukey's post hoc test) was used to compare more than two groups. Two-way analysis of variance followed by Bonferroni's post hoc test was used to determine significant differences in NMDAR-EPSCs and CK2 protein levels at different time points between SNL and control groups and the effects of intrathecal treatment with inhibitors or siRNAs on the paw withdrawal thresholds. The level of significance was set at $P < 0.05$.

Results

Nerve Injury Potentiates NMDAR Activity of Spinal Lamina II, but Not Lamina I, Neurons. It has been shown that most neurons in lamina II are glutamate-releasing excitatory interneurons (Santos et al., 2007). These neurons form a network that plays a critical role in modulating nociceptive information from the primary afferents and controlling the activity of spinal projection neurons (Cervero and Iggo, 1980; Pan et al., 2003). We first determined whether NMDAR activity in the spinal dorsal horn is increased in neuropathic pain caused by peripheral nerve injury. SNL

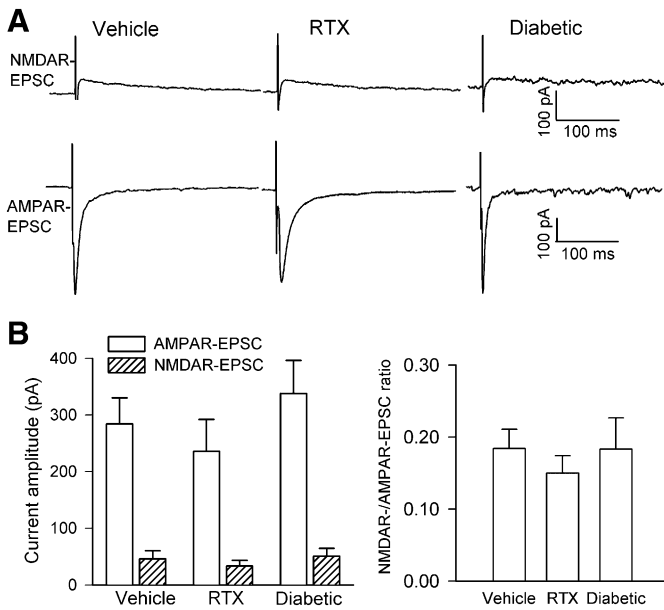


Fig. 4. The NMDAR activity of spinal lamina II neurons does not change in RTX-induced painful neuropathy and diabetic neuropathic pain. (A) Representative traces show evoked NMDAR-EPSCs and AMPAR-EPSCs of a lamina II neuron from one vehicle-treated, one RTX-treated, and one diabetic rat. (B) Summary data show the amplitude of evoked NMDAR-EPSCs and AMPAR-EPSCs (left) and the ratio of NMDAR-EPSCs to AMPAR-EPSCs in lamina II neurons of vehicle-treated ($n = 10$ neurons), RTX-treated ($n = 9$ neurons), and diabetic ($n = 10$ neurons) rats. Error bars represent the S.E.M.

caused a large reduction in the paw withdrawal threshold in response to the pressure stimulus (preinjury, 124.2 ± 2.2 g; SNL, 73.4 ± 2.1 g; $n = 34$ rats, $P < 0.05$) and von Frey filaments (preinjury, 23.04 ± 1.05 g; SNL, 2.04 ± 0.03 g; $n = 34$ rats, $P < 0.05$) 3 weeks after surgery. We recorded monosynaptic NMDAR-EPSCs and AMPAR-EPSCs in lamina II neurons evoked by electrical stimulation of the dorsal root in SNL and sham control rats. Compared with that in sham control rats, SNL caused a large increase in the amplitude of evoked NMDAR-EPSCs 3 weeks, but not 3 days and 7 days, after surgery (Fig. 1, A and B). However, the amplitude of AMPAR-EPSCs did not differ significantly between SNL and control rats. The ratio of NMDAR-EPSCs to AMPAR-EPSCs of lamina II neurons was twice as large in SNL rats as in control rats 3 weeks after surgery (Fig. 1B). In addition, the decay time (τ value) of NMDAR currents was significantly less in SNL rats than in control rats (71 ± 13 milliseconds versus 111 ± 9 milliseconds).

Glutamate released from primary afferent terminals occurs in an activity-dependent manner and activates postsynaptic NMDARs in the spinal dorsal horn neurons. To directly determine whether the postsynaptic NMDAR activity is increased by nerve injury, we recorded NMDAR currents elicited by puff application of $100 \mu\text{M}$ NMDA directly to the recorded lamina II neuron. The amplitude of NMDAR currents of lamina II neurons was much larger in SNL rats than in control rats 3 weeks after surgery (281 ± 45 pA versus 148 ± 24 pA, $P < 0.05$; Fig. 1, C and D).

Laminae I and II of the spinal cord are the central sites of termination of A δ - and C-fiber afferents carrying nociceptive information (Cervero and Iggo, 1980; Pan et al., 2003). Because many lamina I neurons are projection neurons and are

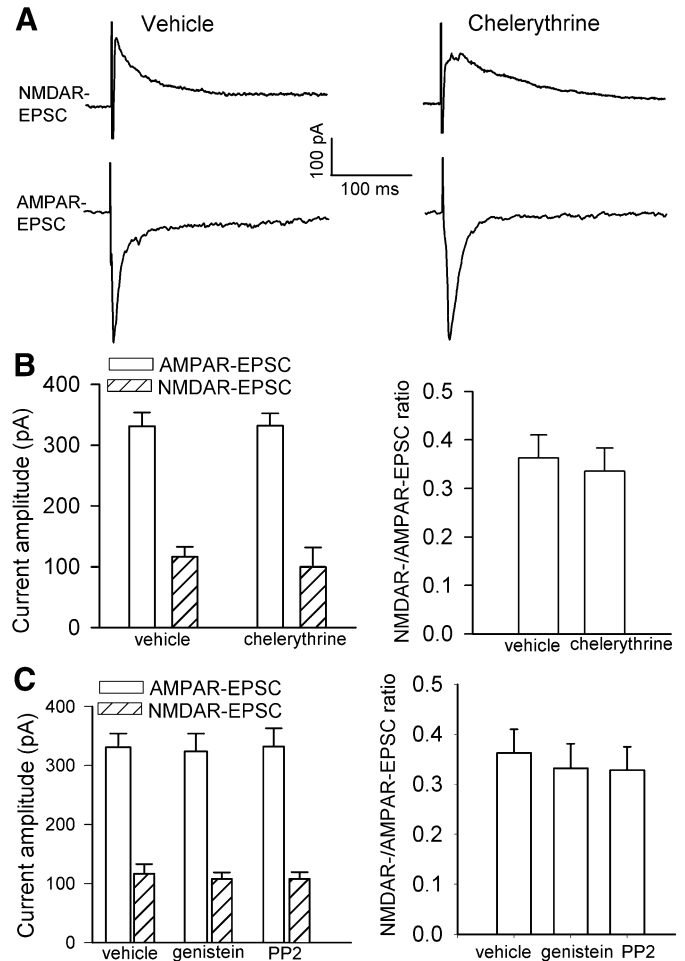


Fig. 5. PKC and protein tyrosine kinase do not contribute significantly to nerve injury-induced increase in NMDAR activity of spinal dorsal horn neurons. (A and B) Original recordings (A) and mean changes (B) in the amplitude of evoked NMDAR-EPSCs and AMPAR-EPSCs of lamina II neurons in SNL rat spinal cord slices treated with vehicle ($n = 16$ neurons) or $10 \mu\text{M}$ chelerythrine ($n = 10$ neurons) 3 weeks after nerve injury. (C) Summary data show the amplitude of evoked NMDAR-EPSCs and AMPAR-EPSCs of lamina II neurons in SNL rat spinal cord slices treated with vehicle ($n = 11$ neurons), $100 \mu\text{M}$ genistein ($n = 12$ neurons), or $1 \mu\text{M}$ PP2 ($n = 8$ neurons). Error bars represent the S.E.M.

also involved in the relay of nociceptive information, we measured monosynaptic NMDAR- and AMPAR-EPSCs induced by the dorsal root stimulation and NMDAR currents elicited by puff application of $100 \mu\text{M}$ NMDA in large-diameter lamina I neurons. The amplitude of NMDAR-EPSCs was significantly smaller in lamina I than in lamina II neurons in control rats (38 ± 6 pA versus 55 ± 6 pA, $P < 0.05$), as was the amplitude of puff NMDA-elicited NMDAR currents (63 ± 11 pA versus 148 ± 24 pA, $P < 0.05$; Figs. 1 and 2). There were no significant differences in the amplitudes of NMDAR-EPSCs and puff NMDA-elicited NMDAR currents of lamina I neurons between SNL rats and the control rats 3 weeks after surgery (Fig. 2). Collectively, these data indicate that peripheral nerve injury potentiates postsynaptic NMDAR activity in spinal lamina II neurons.

Role of GluN2A and GluN2B in Increased NMDAR Activity of Dorsal Horn Neurons by Nerve Injury. Because the reduced decay time of spinal NMDAR currents by nerve injury suggests a possible GluN2 subunit switch, we

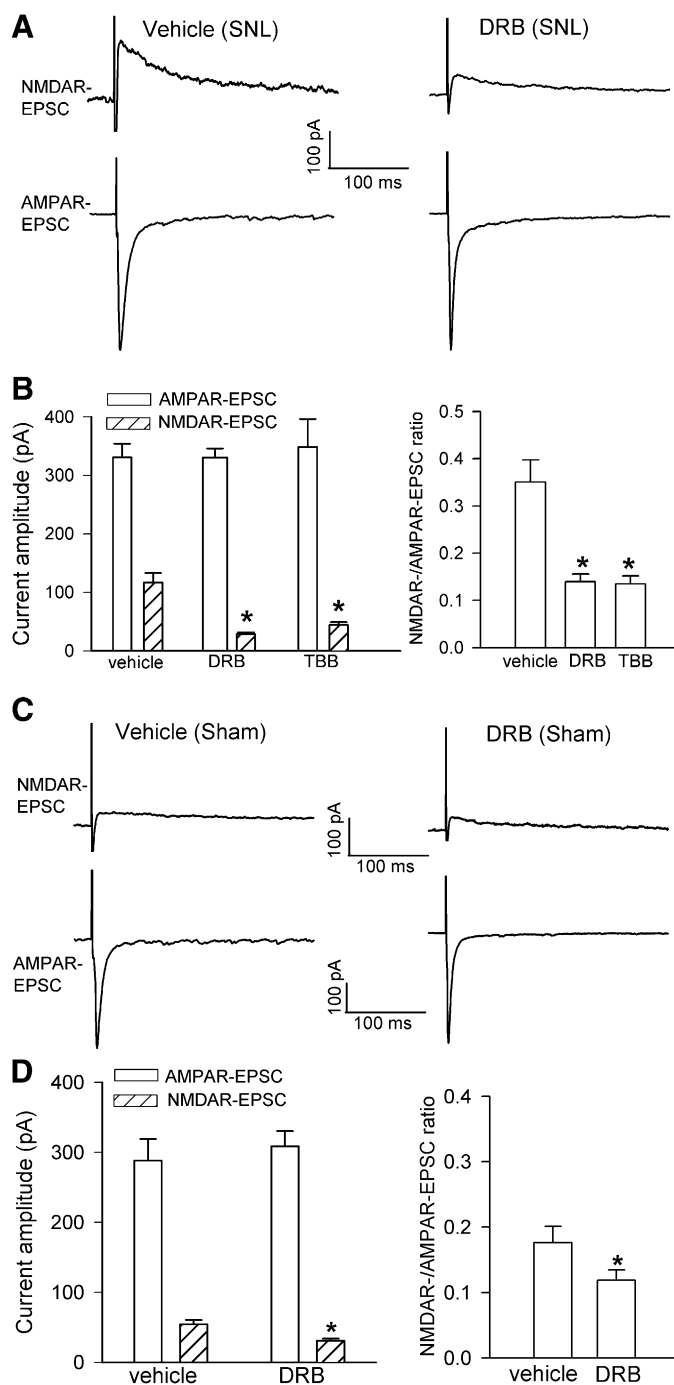


Fig. 6. CK2 contributes to the increased NMDAR activity of spinal dorsal horn neurons caused by nerve injury. (A and B) Representative recordings (A) and mean changes (B) in the amplitude of evoked NMDAR-EPSCs and AMPAR-EPSCs of lamina II neurons in SNL rat spinal cord slices treated with vehicle (dimethylsulfoxide, $n = 16$ neurons), $100 \mu\text{M}$ DRB ($n = 10$ neurons), or $2 \mu\text{M}$ TBB ($n = 10$ neurons). (C and D) Representative recordings (C) and mean changes (D) in the amplitude of evoked NMDAR-EPSCs and AMPAR-EPSCs of lamina II neurons in control rat spinal slices treated with vehicle ($n = 13$ neurons) or $100 \mu\text{M}$ DRB ($n = 11$ neurons). * $P < 0.05$ compared with the vehicle control group. Error bars represent the S.E.M.

next determined the relative contribution of GluN2A and GluN2B subunits to the increased NMDAR activity of lamina II neurons 3 weeks after SNL. We used $0.6 \mu\text{M}$ Ro 25-6981 [($\alpha\text{R},\beta\text{S}$)- α -(4-hydroxyphenyl)- β -methyl-4-(phenylmethyl)-1-piperidinepropanol maleate], a highly specific GluN2B-containing

NMDAR antagonist (Fischer et al., 1997; Zhao et al., 2012), as well as a low concentration of AP5 ($5 \mu\text{M}$), which preferentially blocks GluN2A-containing NMDARs at this concentration (Kimura and Matsuki, 2008; Zhao et al., 2012). In lamina II neurons from sham ($n = 11$ neurons) and SNL ($n = 12$ neurons) rats, Ro 25-6981 caused a similar reduction in the amplitude of NMDAR-EPSCs (Fig. 3). However, further treatment with $5 \mu\text{M}$ AP5 in the presence of Ro 25-6981 produced a significantly larger decrease in the amplitude of NMDAR-EPSCs in SNL rats than in sham rats (Fig. 3). Thus, nerve injury-induced increases in NMDAR activity of lamina II neurons seem to be primarily mediated by GluN2A.

NMDAR Activity of Dorsal Horn Neurons Is Not Altered in Diabetic Neuropathy and Postherpetic Neuralgia Induced by RTX. Although NMDARs in the spinal cord are generally considered important for neuropathic pain conditions, clinical studies suggest that NMDAR antagonists have highly variable effects in different types of neuropathic pain (Zhou et al., 2011b). Therefore, we investigated whether synaptic NMDAR activity of spinal lamina II neurons is altered in postherpetic neuralgia and painful diabetic neuropathy, two clinically important neuropathic pain states that are resistant to treatment with NMDAR antagonists (Nelson et al., 1997; Sang et al., 2002). We used RTX-treated rats as an animal model of postherpetic neuralgia (Pan et al., 2003; Chen and Pan, 2005). Four weeks after treatment, the tactile withdrawal threshold in RTX-treated and vehicle-treated control rats was 2.37 ± 6.46 g and 22.33 ± 4.51 g, respectively ($n = 6$ rats in each group, $P < 0.05$). The amplitude of evoked NMDAR-EPSCs and the ratio of NMDAR-EPSCs to AMPAR-EPSCs in spinal lamina II neurons did not differ significantly between vehicle-treated rats and RTX-treated rats (Fig. 4).

All diabetic rats used for the electrophysiological study developed hyperalgesia, and the pressure withdrawal threshold before and 3 weeks after STZ treatment was 126.35 ± 5.74 g and 78.52 ± 4.87 g, respectively ($n = 6$ rats, $P < 0.05$). In diabetic rats with mechanical hyperalgesia, the amplitude of evoked NMDAR-EPSCs and the ratio of NMDAR-EPSCs to AMPAR-EPSCs in lamina II neurons were similar to those of vehicle-treated rats (Fig. 4). These results suggest that increased spinal NMDAR activity is not associated with postherpetic neuralgia and painful diabetic neuropathy.

PKC and Protein Tyrosine Kinases Do Not Contribute Significantly to Nerve Injury-Induced Increases in Spinal NMDAR Activity. It has been shown that nerve injury increases phosphorylation of NMDARs in the spinal cord (Gao et al., 2005). However, the protein kinases responsible for increased NMDAR activity in the spinal cord after nerve injury remain unclear. PKC can increase NMDAR activity by reducing the Mg^{2+} block of the NMDAR channels in dissociated sensory neurons (Chen and Huang, 1992). Chelerythrine is a specific PKC inhibitor (Herbert et al., 1990), and we have shown that $10 \mu\text{M}$ chelerythrine reduces NMDAR activity in the spinal cord potentiated by repeated opioid administrations (Zhao et al., 2012). However, incubation with chelerythrine ($10 \mu\text{M}$ for 2 to 3 hours) had no significant effect on the amplitude of evoked NMDAR-EPSCs or the ratio of NMDAR-EPSCs to AMPAR-EPSCs in lamina II neurons 3 weeks after SNL (Fig. 5, A and B).

Protein tyrosine kinase Src has also been shown to enhance the NMDAR activity in cultured spinal cord neurons (Yu et al.,

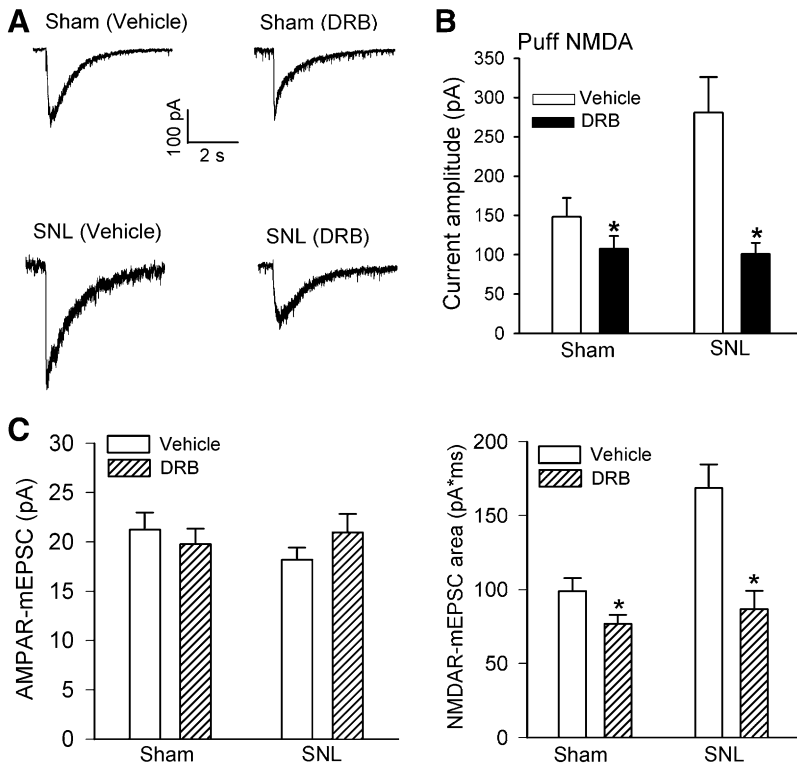


Fig. 7. CK2 plays a critical role in nerve injury-induced increase in the postsynaptic NMDAR activity of spinal dorsal horn neurons. (A and B) Original traces (A) and mean changes (B) in the NMDAR currents elicited by puff NMDA to lamina II neurons in SNL rat spinal slices treated with vehicle (dimethylsulfoxide, $n = 19$ neurons) or DRB ($n = 23$ neurons). (C) Summary data show AMPAR- and NMDAR-mediated mEPSCs of lamina II neurons in spinal cord slices treated with vehicle or DRB in sham control and SNL rats. * $P < 0.05$ compared with the vehicle control group. Error bars represent the S.E.M.

1997). Genistein, a broad-spectrum protein tyrosine kinase inhibitor, inhibits the activity of protein tyrosine kinases, including Src (Akiyama et al., 1987; Devary et al., 1992; Bare et al., 1995). However, treatment of spinal cord slices of SNL rats with genistein (100 μM for 2 to 3 hours) failed to significantly inhibit the potentiated NMDAR-EPSCs in lamina II neurons 3 weeks after SNL (Fig. 5C). PP2 is a selective Src family tyrosine kinase inhibitor (Hanke et al., 1996). In separate lamina II neurons from SNL rats, treatment with PP2 (1 μM for 2 to 3 hours) had little effect on the amplitude of evoked NMDAR-EPSCs or the ratio of NMDAR-EPSCs to AMPAR-EPSCs (Fig. 5C). These data suggest that PKC and protein tyrosine kinases do not play a major role in increased spinal NMDAR activity caused by nerve injury.

Nerve Injury Augments NMDAR Activity of Spinal Dorsal Horn Neurons through CK2. In the hippocampus and hypothalamus, CK2 activation can increase NMDAR activity (Lieberman and Mody, 1999; Ye et al., 2012). We thus determined whether CK2 contributes to enhanced postsynaptic NMDAR activity of lamina II neurons in SNL rats. Both DRB and TBB are selective inhibitors of CK2 and competitively inhibit the binding of ATP and GTP as phosphate donors of CK2 (Sarno et al., 2001, 2002). DRB is considered to be a highly specific inhibitor of CK2, with no effect on PKC, protein kinase A, Ca^{2+} /calmodulin-dependent protein kinase II, or the Src tyrosine kinase (Pinna, 1990). The concentration of DRB (100 μM) used in this study has been shown to inhibit CK2 activity in rat brain slices (Lieberman and Mody, 1999; Ye et al., 2012). In addition, we used TBB, a structurally distinct, specific CK2 inhibitor. The 50% inhibitory concentration of TBB is 0.9 μM (Sarno et al., 2001, 2002). Incubation with DRB or TBB (2 μM) for 2 to 3 hours completely normalized the amplitude of evoked NMDAR-EPSCs and the ratio of NMDAR-EPSCs to AMPAR-EPSCs in dorsal horn

neurons 3 weeks after SNL (Fig. 6, A and B). DRB also substantially reduced the amplitude of NMDAR currents elicited by puff application of NMDA and the NMDAR-mediated component of mEPSCs in lamina II neurons of SNL rats (Figs. 6, C and D, and 7). Similar DRB treatment slightly but significantly reduced the amplitude of NMDAR-EPSCs and puff NMDA-elicited NMDAR currents in lamina II neurons of control rats (Figs. 6, C and D, and 7, A and B). These findings indicate that CK2 plays a key role in increased postsynaptic NMDAR activity in the spinal dorsal horn induced by nerve injury.

CK2 and Calcineurin Dynamically Regulate NMDAR Activity in the Spinal Cord. The NMDAR activity and phosphorylation state may be dynamically controlled by a balance between the activity of protein kinases and protein phosphatases. Because the calcium-dependent protein phosphatase calcineurin (PP2B) negatively affects NMDAR activity in the brain (Lieberman and Mody, 1994; Tong et al., 1995), we investigated whether calcineurin participates in the control of NMDAR activity in the spinal cord. Incubation with the specific calcineurin inhibitor FK-506 (1 μM for 2 to 3 hours) (Liu et al., 1991; Li et al., 2012a) caused a large increase in the amplitude of evoked NMDAR-EPSCs, but not AMPAR-EPSCs, in lamina II neurons of control rats (Fig. 8, A and B). The magnitude of NMDAR-EPSCs increased by FK-506 in control rats was similar to that of SNL rats.

Okadaic acid is the protein phosphatase PP1/PP2A inhibitor (Haystead et al., 1989), and 100 nM okadaic acid is known to inhibit PP1/PP2A activity in brain slices (Garver et al., 1995; Chergui et al., 2005). However, treatment of spinal cord slices with okadaic acid (100 nM for 2 to 3 hours) had no significant effect on the amplitude of AMPAR-EPSCs and NMDAR-EPSCs in lamina II neurons of sham control rats (Fig. 8B). In addition, in lamina II neurons of SNL rats, FK-506

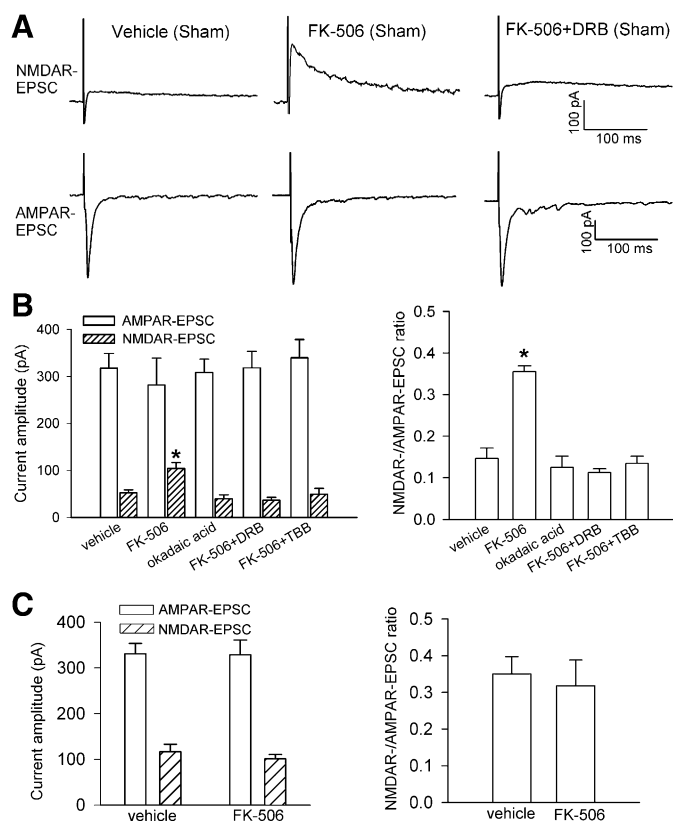


Fig. 8. NMDAR activity of spinal dorsal horn neurons is dynamically controlled by CK2 and calcineurin. (A and B) Representative recordings (A) and mean changes (B) in the amplitude of evoked NMDAR-EPSCs and AMPAR-EPSCs of lamina II neurons in control rat spinal slices treated with vehicle (dimethylsulfoxide, $n = 13$ neurons), $1 \mu\text{M}$ FK-506 ($n = 10$ neurons), $0.1 \mu\text{M}$ okadaic acid ($n = 12$ neurons), FK-506 plus DRB ($n = 10$ neurons), or FK-506 plus TBB ($n = 10$ neurons). (C) Mean changes in the amplitude of evoked NMDAR-EPSCs and AMPAR-EPSCs of lamina II neurons in SNL rat spinal cord slices treated with vehicle ($n = 16$ neurons) or $1 \mu\text{M}$ FK-506 ($n = 14$ neurons). * $P < 0.05$ compared with the vehicle control group. Error bars represent the S.E.M.

treatment had no further effect on the increased amplitude of NMDAR-EPSCs (Fig. 8C).

If CK2 controls NMDAR activity through phosphorylation, then inhibition of endogenous CK2 should prevent the calcineurin inhibitor FK-506 from potentiating the NMDAR activity in the spinal cord (Chen et al., 2014; Hu et al., 2014). Indeed, concurrent treatment with FK-506 with DRB or TBB abolished the FK-506-induced potentiation of NMDAR-EPSCs in dorsal horn neurons of control rats (Fig. 8, A and B). Interestingly, incubation with FK-506 alone did not significantly change the already increased amplitude of NMDAR-EPSCs of lamina II neurons 3 weeks after SNL (Fig. 8C). These data strongly suggest that the NMDARs in the spinal dorsal horn are reciprocally controlled by CK2 and calcineurin and that the potentiated spinal NMDAR activity caused by nerve injury may result from the imbalance between the activity of CK2 and calcineurin.

Nerve Injury Increases CK2 Protein Levels in the Spinal Cord. The α/α' catalytic subunit of CK2 confers enzymatic activity, whereas the β subunit confers stability and specificity to the holoenzyme and regulates overall CK2 enzymatic activity (Blanquet, 2000). To determine whether nerve injury upregulates CK2 subunits in the spinal cord, we

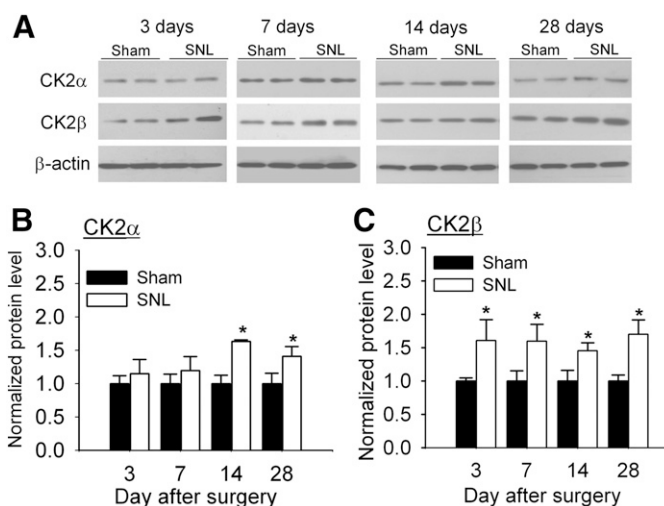


Fig. 9. Nerve injury increases CK2 α and CK2 β protein levels in the dorsal spinal cord. (A–C) Immunoblotting gel images (A) and quantification of CK2 α (approximately 45 kDa) (B), and CK2 β (approximately 25 kDa) (C) protein levels in the dorsal spinal cord ipsilateral to SNL and contralateral (sham) controls ($n = 6$ rats in each group). The dorsal spinal cord tissues were obtained 3, 7, 14, and 28 days after surgery. The CK2 α and CK2 β protein amounts were normalized to β -actin (loading controls) in each sample. * $P < 0.05$ compared with the contralateral control. Error bars represent the S.E.M.

measured the protein levels of CK2 α and CK2 β in the dorsal spinal cord at days 3, 7, 14, and 28 after SNL. Nerve injury caused a persistent increase in the CK2 β level in the lumbar dorsal spinal cord ipsilateral to SNL compared with the contralateral (sham) controls starting at day 3 (Fig. 9). SNL also significantly increased the CK2 α level in the dorsal spinal cord at days 14 and 28 after SNL surgery (Fig. 9). These data indicate that nerve injury upregulates CK2 in the spinal cord, which may be involved in the transition from acute to chronic neuropathic pain.

CK2 at the Spinal Level Contributes to Allodynia and Hyperalgesia Induced by Nerve Injury. To determine whether CK2 at the spinal level contributes to pain hypersensitivity induced by SNL, we tested the effect of intrathecal injection of TBB or DRB on tactile allodynia and mechanical hyperalgesia 3 to 4 weeks after SNL. Drugs for intrathecal injections were dissolved in 30% dimethylsulfoxide in saline and administered in a volume of $5 \mu\text{l}$, followed by a $10 \mu\text{l}$ flush with normal saline. TBB (100, 200, and 500 ng) produced a dose-dependent and long-lasting reduction in tactile allodynia and mechanical hyperalgesia, measured with application of von Frey filaments and the noxious pressure stimulus to the left hindpaw of SNL rats ($n = 7$ to 8 rats in each group; Fig. 10, A and B). Administration of TBB (500 ng) did not significantly alter the nociceptive mechanical threshold in control rats (Fig. 10B).

Because SNL persistently increased the protein level of CK2 β in the spinal cord, we intrathecally injected siRNA to knock down CK2 β expression to examine the contribution of spinal CK2 β to pain hypersensitivity induced by SNL. We screened and selected two CK2 β -specific siRNAs and conjugated them to the chitosan nanoparticles (Cai et al., 2009; Zhou et al., 2012). Quantitative PCR analysis revealed a large reduction (approximately 50%) in the mRNA level of CK2 β in both sides of the dorsal spinal cord of SNL rats treated intrathecally with CK2 β -specific siRNA ($5 \mu\text{g}/\text{d}$ for 3 days).

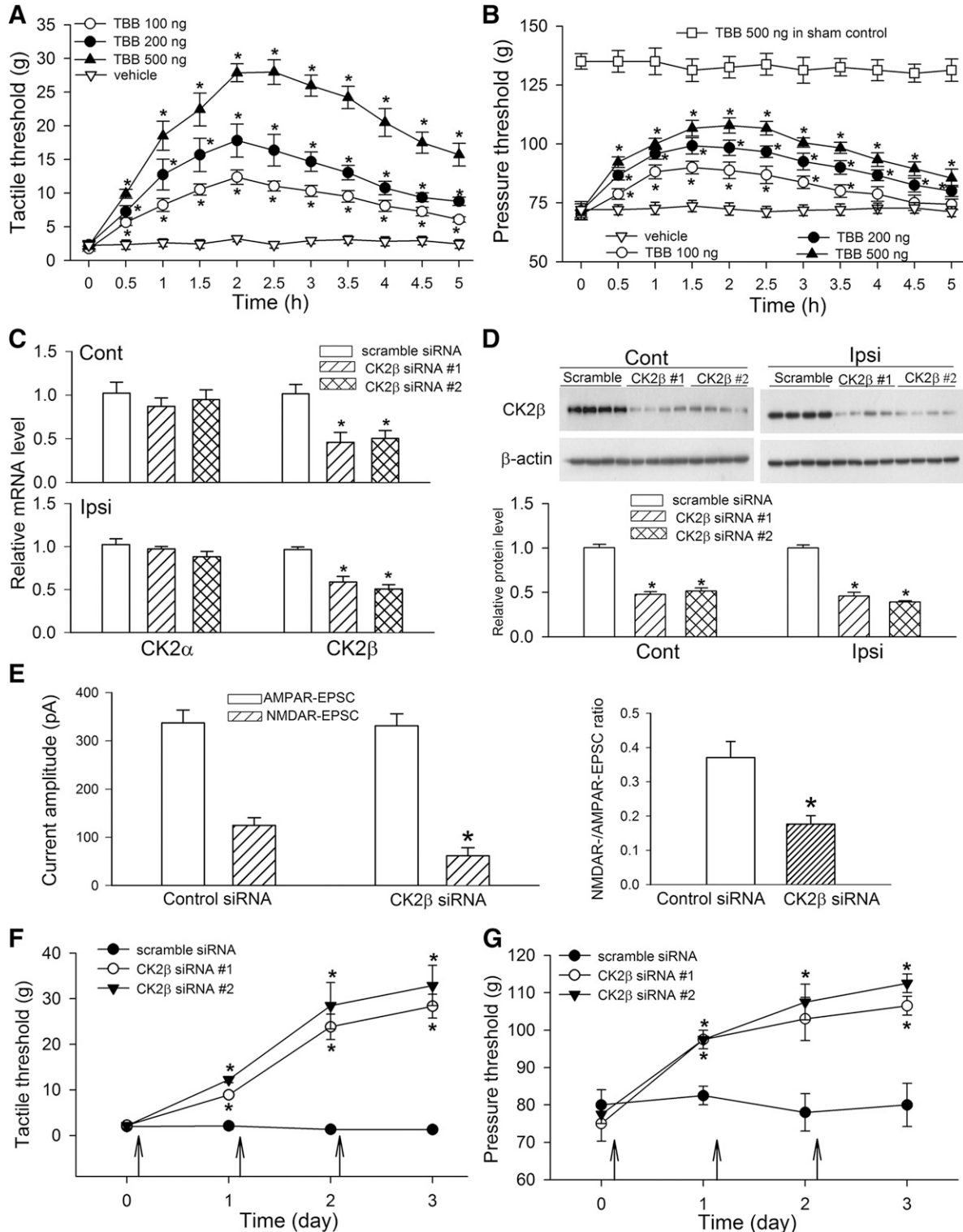


Fig. 10. Inhibition of CK2 and siRNA knockdown of CK2 β expression at the spinal level reduces pain hypersensitivity induced by nerve injury. (A and B) Time course of the effects of intrathecal injection of 100, 200, and 500 ng TBB and vehicle (10% dimethylsulfoxide) on the withdrawal threshold measured with von Frey filaments (A) and a pressure stimulus (B) in rats 3 weeks after SNL or sham surgery ($n = 7$ to 8 rats per group). (C) Quantitative PCR analysis of CK2 α and CK2 β mRNA levels in the dorsal spinal cord ipsilateral (Ipsi) and contralateral (Cont) to nerve ligation of SNL rats treated with the two CK2 β -specific siRNAs and a scramble control siRNA ($n = 4$ samples in each group). (D) Immunoblotting gel images (top) and mean changes (bottom) show the CK2 β protein levels in the dorsal spinal cord of SNL rats treated with the two CK2 β -specific siRNAs and a scramble control siRNA ($n = 6$ samples in each group). (E) Mean changes in the amplitude and ratio of NMDAR-EPSCs to AMPAR-EPSCs of lamina II neurons recorded from SNL rats treated with the scramble control siRNA ($n = 11$ neurons) or CK2 β -specific siRNA ($n = 12$ neurons). (F and G) Changes in the paw withdrawal threshold measured with von Frey filaments (F) and a pressure stimulus (G) ($n = 7$ to 8 rats in each group) of SNL rats treated intrathecally with the two CK2 β -specific siRNAs and a scramble control siRNA ($n = 7$ rats in each group). Threshold tests were performed before siRNA injection each day (indicated by arrows). * $P < 0.05$ compared with the respective predrug control or the control siRNA group. Error bars represent the S.E.M.

However, the CK2 α mRNA level was not significantly affected by treatment with CK2 β -specific siRNA (Fig. 10C). Furthermore, treatment with the CK2 β -specific siRNA induced a reduction of approximately 50% in the CK2 β protein level in the dorsal spinal cord and significantly attenuated the amplitude of NMDAR-EPSCs of lamina II neurons in SNL rats (Fig. 10, D and E). In addition, intrathecal injection of either of the CK2 β -specific siRNAs, but not the control scrambled siRNA, for 3 consecutive days gradually and significantly increased the paw withdrawal threshold in response to application of von Frey filaments and the noxious pressure stimulus in SNL rats (Fig. 10, F and G). These data indicate that CK2 at the spinal level contributes to pain hypersensitivity induced by nerve injury.

Discussion

Although the importance of NMDARs as a therapeutic target for certain types of neuropathic pain has been well recognized, very few studies have directly examined changes in NMDAR activity in the spinal dorsal horn in different neuropathic pain conditions. We found that nerve injury induced a substantial increase in the amplitudes of NMDAR-EPSCs and postsynaptic NMDAR currents elicited by NMDA puff application in spinal lamina II neurons 3 weeks, but not 3 and 7 days, after SNL. Interestingly, we found that the postsynaptic NMDAR currents were much smaller in lamina I than in lamina II neurons and that nerve injury had no significant effect on NMDAR activity of lamina I neurons. These data indicate that nerve injury-induced increases in postsynaptic NMDAR activity in the spinal dorsal horn are time dependent and lamina specific. By analyzing the decay time of NMDAR-EPSCs and using selective GluN2A and GluN2B blockers, we showed that increased NMDAR activity of lamina II neurons by nerve injury is primarily mediated by GluN2A. More importantly, we showed that the NMDAR activity of spinal dorsal horn neurons is not significantly altered in rat models of diabetic neuropathic pain or postherpetic neuralgia. These data are consistent with clinical studies showing that NMDAR antagonists produce long-lasting relief in patients with nerve injury-induced chronic pain (Rabben et al., 1999; Correll et al., 2004; Schwartzman et al., 2009) but have limited analgesic effects for patients with painful diabetic neuropathy or postherpetic neuralgia (Nelson et al., 1997; Sang et al., 2002). Our study provides strong evidence that neuropathic pain conditions with different etiologies do not share the same mechanisms and that increased NMDAR activity in the spinal dorsal horn is distinctly associated with traumatic nerve injury.

Activity-dependent synaptic plasticity at the spinal cord level is fundamentally important to the development of neuropathic pain (Wang et al., 2007; Zhou et al., 2011a, 2012). Although nerve injury increases NMDAR phosphorylation in the spinal cord (Gao et al., 2005), the exact protein kinases responsible for the increased NMDAR activity in the spinal cord caused by nerve injury remain uncertain. The protein kinases that can increase NMDAR activity include PKC (Chen and Huang, 1992) and protein tyrosine kinase (Yu et al., 1997). For example, intracellular application of PKC increases NMDAR currents in isolated trigeminal neurons (Chen and Huang, 1992). Contrary to our prediction, we found that inhibition of PKC with chelerythrine had no significant

effect on the amplitude of NMDAR-EPSCs or the ratio of NMDAR-EPSCs to AMPAR-EPSCs in dorsal horn neurons of SNL rats. Although the protein tyrosine kinase Src can increase NMDAR activity in cultured fetal spinal cord neurons (Yu et al., 1997), there is no direct electrophysiological evidence showing that tyrosine kinases are responsible for the nerve injury-induced increase in the NMDAR currents of spinal dorsal horn neurons. We found that inhibition of tyrosine kinases with genistein or PP2 failed to significantly alter augmented NMDAR activity of dorsal horn neurons in SNL rats. Unlike the previous work using dissociated or cultured neurons, this study was conducted using spinal cord slice preparation in which the primary afferent nerve (dorsal root) input to dorsal horn neurons remains largely intact. Therefore, despite the fact that PKC and protein tyrosine kinases can increase phosphorylation and activity of NMDARs, they do not play a major role in the increased NMDAR activity of spinal dorsal horn neurons caused by nerve injury.

An important finding of our study is that CK2 contributes critically to the nerve injury-induced increase in the NMDAR activity of spinal dorsal horn neurons. CK2 is a highly conserved serine/threonine protein kinase and is widely expressed in the central nervous system (Blanquet, 2000). CK2 is specifically enriched at the postsynaptic densities, which are crucial for synaptic plasticity (Lieberman and Mody, 1999; Soto et al., 2004). It has been shown that CK2 activity rapidly increases after the induction of long-term potentiation in the brain (Charriaut-Marlangue et al., 1991). In this study, we found that inhibition of CK2 with DRB or TBB completely normalized the amplitude of NMDAR-EPSCs and NMDAR currents elicited by puff application of NMDA in the spinal dorsal horn of SNL rats. Inhibition of CK2 had only a minor effect on the NMDAR activity of dorsal horn neurons in control rats. Thus, CK2 not only contributes to the increased spinal NMDAR activity caused by nerve injury but also tonically regulates NMDAR activity in the physiologic condition.

In this study, we found that nerve injury caused an early and persistent increase in the CK2 β protein level, but the CK2 α protein level in the dorsal spinal cord increased significantly much later after nerve injury. Our results indicate that nerve injury upregulates CK2, which plays a key role in the augmented synaptic NMDAR activity in the spinal dorsal horn. Similar to the delayed increase in spinal CK2 activity (reflected in a combined increase in CK2 α and CK2 β protein levels), a significant increase in spinal NMDAR activity was only evident 3 weeks after SNL. Thus, the CK2-mediated increases in spinal synaptic NMDAR activity may contribute to the mechanisms underlying the transition of acute pain to chronicity after nerve injury. However, we do not know how CK2 activity is increased and how CK2 potentiates GluN2A-mediated NMDAR activity after nerve injury. CK2 can modulate NMDAR activity directly or indirectly. For example, CK2 may increase NMDAR activity through the phosphorylation of GluN2B Ser¹⁴⁸⁰ (to decrease GluN2B surface expression) (Chung et al., 2004) or the NMDAR scaffolding protein postsynaptic density protein-95 (also called synapse-associated protein-90) (Soto et al., 2004). In addition, calmodulin, a substrate of CK2 (Sacks et al., 1992), can inhibit the duration of NMDAR channel openings through binding to the NR1 subunit (Ehlers et al., 1996; Krupp et al., 1999). CK2 may phosphorylate calmodulin, resulting in an increase in NMDAR activity (Ehlers et al., 1996).

Protein phosphorylation by the coordinated activities of protein kinases and phosphatases is central to many signal transduction pathways. Under the physiologic condition, synaptic NMDARs may fluctuate between phosphorylated and dephosphorylated forms. The protein phosphatases that negatively regulate the function of NMDARs include calcineurin (Lieberman and Mody, 1994; Tong et al., 1995) and protein tyrosine phosphatase (Wang and Salter, 1994). However, how the cycle of phosphorylation/dephosphorylation shifts in neuropathic pain is unclear. We found that the calcineurin inhibitor FK-506 profoundly increased NMDAR activity of dorsal horn neurons in control rats, but the protein phosphatase PP1/PP2A inhibitor okadaic acid did not. Interestingly, FK-506 had no effect on the already increased NMDAR activity of dorsal horn neurons in SNL rats. Furthermore, CK2 inhibition blocked the FK-506-induced potentiation of NMDAR currents in dorsal horn neurons of control rats. It has been shown that the calcineurin level in the spinal cord is reduced after peripheral nerve injury (Miletic et al., 2002); the imbalance between the levels of CK2 and calcineurin could play a critical role in increased spinal NMDAR activity in neuropathic pain. It thus is possible that the phosphorylation/dephosphorylation cycle of NMDARs in the spinal dorsal horn is shifted to a predominantly phosphorylated state through increased CK2 activity after nerve injury. In addition, calcineurin inhibitors, such as cyclosporin A and FK-506 (tacrolimus), are the most commonly used immunosuppressive drugs to prevent the rejection of transplanted organs and tissues. However, these drugs can cause unexplained persistent pain and pain hypersensitivity, often referred to as calcineurin inhibitor-induced pain syndrome (Grotz et al., 2001; Collini et al., 2006). Indeed, systemic administration of FK-506 can cause long-lasting pain hypersensitivity through potentiating NMDAR activity in the spinal dorsal horn (Chen et al., 2014). Thus, inhibition of CK2 and NMDAR function may reduce calcineurin inhibitor-induced pain syndrome (Chen et al., 2014; Hu et al., 2014).

Another salient finding of our study is that CK2 at the spinal level plays a key role in nerve injury-induced pain hypersensitivity. We found that intrathecal injection of CK2 inhibitors markedly reduced tactile allodynia and mechanical hyperalgesia in SNL rats. By contrast, inhibition of spinal CK2 had no significant effect on mechanical nociception in control rats. The CK2 β subunit is involved in assembly of functional tetrameric CK2 complexes and in regulation of CK2 catalytic activity and substrate specificity (Blanquet, 2000). Because nerve injury caused a large and persistent increase in CK2 β protein levels in the spinal cord, we sought to use CK2 β -specific siRNAs to determine the contribution of spinal CK2 β to nerve injury-induced pain hypersensitivity. Chitosan is an ideal nonviral vector for siRNA delivery because its protonated amine groups allow transport across cellular membranes and subsequent endocytosis into the neurons. We demonstrated that the CK2 β siRNA conjugated to chitosan specifically knocked down CK2 β expression levels in the spinal cord and attenuated SNL-induced increases in synaptic NMDAR currents. Intrathecal administration of the CK2 β -specific siRNA significantly reversed tactile allodynia and mechanical hyperalgesia in SNL rats. These data reinforce the pivotal role of CK2 upregulation in the spinal cord in increased NMDAR activity and neuropathic pain state caused by nerve injury.

In conclusion, we provide novel evidence for the prominent role of CK2 in the increased spinal NMDAR activity and pain hypersensitivity induced by nerve injury. Upregulation of CK2 in the spinal cord after nerve injury may contribute to the transition from acute to chronic pain by potentiating and sustaining the elevated activity of NMDARs. Our new findings are critical not only to the improvement of our understanding of the molecular mechanisms of neuropathic pain but also to the development of new strategies to treat neuropathic pain. NMDAR channel blockers, such as ketamine, produce intolerable adverse effects due to the involvement of NMDARs in many physiologic functions. The initial clinical trials show that CK2 inhibitors are safe for human use. Our study suggests that CK2 could be a new target for treating nerve injury-induced neuropathic pain and calcineurin inhibitor-induced pain syndrome by reducing potentiated NMDAR activity without directly impairing the NMDAR channel function.

Authorship Contributions

Participated in research design: Pan.

Conducted experiments: S.-R. Chen, Zhou, Byun, H. Chen.

Performed data analysis: S.-R. Chen, Zhou, Byun, Pan.

Wrote or contributed to the writing of the manuscript: S.-R. Chen, Pan.

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