

BDNF contributes to the development of neuropathic pain by induction of spinal long-term potentiation via SHP2 associated GluN2B-containing NMDA receptors activation in rats with spinal nerve ligation



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ABSTRACT

The pathogenic mechanisms underlying neuropathic pain still remain largely unknown. In this study, we investigated whether spinal BDNF contributes to dorsal horn LTP induction and neuropathic pain development by activation of GluN2B-NMDA receptors via Src homology-2 domain-containing protein tyrosine phosphatase-2 (SHP2) phosphorylation in rats following spinal nerve ligation (SNL). We first demonstrated that spinal BDNF participates in the development of long-lasting hyperexcitability of dorsal horn WDR neurons (i.e. central sensitization) as well as pain allodynia in both intact and SNL rats. Second, we revealed that BDNF induces spinal LTP at C-fiber synapses via functional up-regulation of GluN2B-NMDA receptors in the spinal dorsal horn, and this BDNF-mediated LTP-like state is responsible for the occlusion of spinal LTP elicited by subsequent high-frequency electrical stimulation (HFS) of the sciatic nerve in SNL rats. Finally, we validated that BDNF-evoked SHP2 phosphorylation is required for subsequent GluN2B-NMDA receptors up-regulation and spinal LTP induction, and also for pain allodynia development. Blockade of SHP2 phosphorylation in the spinal dorsal horn using a potent SHP2 protein tyrosine phosphatase inhibitor NSC-87877, or knockdown of spinal SHP2 by intrathecal delivery of SHP2 siRNA, not only prevents BDNF-mediated GluN2B-NMDA receptors activation as well as spinal LTP induction and pain allodynia elicitation in intact rats, but also reduces the SNL-evoked GluN2B-NMDA receptors up-regulation and spinal LTP occlusion, and ultimately alleviates pain allodynia in neuropathic rats. Taken together, these results suggest that the BDNF/SHP2/GluN2B-NMDA signaling cascade plays a vital role in the development of central sensitization and neuropathic pain after peripheral nerve injury.

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Abbreviations: ACC, the anterior cingulate cortex; AMPAR, a-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor; ANOVA, analysis of variance; AUC, area under the time-course curve; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element-binding protein; ERK, extracellular signal-regulated kinase; GluN2B-NMDA receptors, GluN2B-containing N-methyl-D-aspartate receptors; HFS, high-frequency electrical stimulation; LTP, long-term potentiation; LV-shSHP2, lentivirus-short hairpin SHP2; MAPK, mitogen-activated protein kinase; NMDA receptors, N-methyl-D-aspartate receptors; NS, normal saline; PSD, postsynaptic density; PWT, paw withdrawal threshold; RTK, receptor tyrosine kinase; SHP2, Src homology-2 (H2) domain-containing protein tyrosine phosphatase-2; SNL, spinal nerve ligation; TrkB, tropomyosin related kinase B; WDR neurons, wide dynamic range neurons.

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Introduction

The mechanisms underlying the development of neuropathic pain are still not fully understood. Previously, we and others have reported that sensory information processing in the spinal dorsal horn appears to undergo significant plastic changes e.g. long-term potentiation (LTP) in synaptic efficacy following peripheral nerve injury or inflammation (Park et al., 2011; Sandkuhler and Liu, 1998; Xing et al., 2007; Yang et al., 2014), which is well accepted as the underlying mechanisms of central sensitization and post-injury pain hypersensitivity (Ji et al., 2003; Latremoliere and Woolf, 2009; Sandkuhler, 2007; Woolf, 2011). Moreover, we have previously discovered that activation of spinal GluN2B-containing N-methyl-D-aspartate (GluN2B-NMDA) receptors is required for the induction of dorsal horn LTP at C-fiber synapses after nerve injury, implying GluN2B-dependent LTP in the spinal dorsal horn plays a crucial role in the development of long-lasting spinal neurons hyperexcitability (i.e. central sensitization) and neuropathic pain (Qu et al., 2009). In a parallel study, we have demonstrated that increased spinal brain-derived neurotrophic factor (BDNF) contributes to the

pathogenesis of neuropathic pain by activation of GluN2B-NMDA receptors in rats with spinal nerve ligation (SNL) (Geng et al., 2010). However, the cellular and molecular mechanisms associated with such a role for BDNF still remain unclear.

Several lines of evidence have shown that in some brain regions BDNF plays a key role in activity-dependent LTP, a synaptic model of memory storage (Escobar et al., 2003; Leal et al., 2014; Lu et al., 2008; Meis et al., 2012; Sakata et al., 2013; Ying et al., 2002), and the GluN2B-NMDA receptor is considered as one of the targets for BDNF-induced modulation of synaptic plasticity (Caldeira et al., 2007b; Carreno et al., 2011; Kim et al., 2006; Levine and Kolb, 2000; Lin et al., 1998; Otis et al., 2014; Yamada and Nabeshima, 2004). In the rat spinal dorsal horn, it is also found that the BDNF-induced LTP at C-fiber synapses is involved in the pathogenesis of mechanical hypersensitivity following peripheral nerve injury (Zhou et al., 2008, 2011). We here hypothesized that induction of spinal LTP at C-fiber synapses via activation of GluN2B-NMDA receptors would be the underlying mechanism by which the increased BDNF in the spinal dorsal horn plays its roles in the development of neuropathic pain following nerve injury.

BDNF, a member of the neurotrophins family, should bind to and activate tropomyosin related kinase B (TrkB), a receptor tyrosine kinase, to exert its effects (Park and Poo, 2013). SHP2, a Src homology-2 (H2) domain-containing protein tyrosine phosphatase-2, acting as a major regulator of receptor tyrosine kinase (Grossmann et al., 2010), has been implicated in numerous neurotrophin signaling (Case et al., 1994; Matozaki et al., 2009; Ohnishi et al., 1999; Stein-Gerlach et al., 1998), including BDNF/TrkB signaling pathway (Araki et al., 2000; Easton et al., 2006; Neel et al., 2003; Okada et al., 1996). In an in vitro study, Lin and colleagues have reported that SHP2 participates in BDNF-mediated GluN2B-NMDA receptors signaling at the postsynaptic site, and BDNF enhances association of SHP2 with the NMDA receptor subunit GluN2B in the cortical postsynaptic density (PSD) (Lin et al., 1999). Recently, Peng et al. have revealed that phosphorylation of spinal SHP2 via signal regulatory protein alpha 1 (SIRP α 1) induces SIRP α 1-SHP2 interaction, which subsequently triggers SHP2/PSD-95/GluN2B signaling, and thereby playing a role in neuropathic pain development in a rat model (Peng et al., 2012).

In this study, we investigated whether spinal BDNF contributes to dorsal horn LTP induction and neuropathic pain development by activation of GluN2B-NMDA receptors via SHP2 phosphorylation in rats following SNL surgery. We present valid evidence showing that the BDNF/SHP2/GluN2B-NMDA signaling cascade in the spinal dorsal horn plays a vital role in the induction of spinal LTP as well as in the development of central sensitization and neuropathic pain after peripheral nerve injury.

Materials and methods

Chemicals, recombinant lentivirus, and animals

BDNF (Sigma-Aldrich, St. Louis, MO) was first dissolved as a concentrated stock solution (5.0 μ g/ml) in 0.9% sterile saline (e.g. normal saline, NS). TrkB-Fc (R&D systems, Minneapolis, USA) and IgG (Sigma) were dissolved in 0.01 M phosphate buffer saline (PBS) containing 0.1% bovine serum albumin (BSA) as a 100 mg/ml store solution. Ifenprodil (Sigma), NSC-87877 (Tocris Bioscience, Bristol, UK), genistein (Sigma) and its inactive analogue genistin (Calbiochem, USA) were dissolved in dimethyl sulphoxide (DMSO, sigma, St. Louis, MO, USA) to make a stock concentration of 50 mM, aliquoted in small volumes and stored at -20 °C. The stock solution was subsequently diluted with sterile normal saline to make desired final concentrations immediately before administration. The final concentration of DMSO was <0.5%.

Recombinant lentivirus-short hairpin SHP2 (LV-shSHP2) was constructed by Genechem (Shanghai, China). Briefly, the plasmids expressed shSHP2-GFP was used to produce lentivirions. Lentivirus-short hairpin RNA (shRNA) was generated using sense small interfering RNA sequence targeting SHP2 (GeneBank number NM-001177593.1):

small interfering SHP2 TTAGGAACGTCGATGTAC. The scrambled sequence LV-GFP was used as negative control. To minimize off-target effects, a BLAST homology search (based on sense and antisense sequences) was systematically performed to ensure that a single mRNA sequence was targeted (<http://www.ncbi.nlm.nih.gov>). Lentiviral vector is pGV118 (Genechem, Shanghai, China) with U6 promoter. The short hairpin RNAs were cloned into lentivirus vectors. Construction and production of lentivirions were completed by Genechem. The final titer of recombinant virus was around 1.0×10^9 transducing units (TU)/ml.

Male Sprague-Dawley rats weighing 200–250 g at the beginning of the experiment were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center. The rats were housed in separated cages with free access to food and water. The room temperature was kept at 24 ± 1 °C under natural light-dark cycle. All animal experimental procedures were carried out in accordance with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the Animal Care and Use Committee of Peking University. The behavioral experimenters were kept blind from the groupings of the rats. A total of 443 animals were used in our present study.

Spinal nerve ligation (SNL)

Under general anesthesia with chloral hydrate (0.3 g/kg, intraperitoneally, i.p.), the left lumbar 5 (L5) spinal nerves distal to the dorsal root ganglia were tightly ligated with 4-0 silk sutures as described by Kim and Chung (Kim and Chung, 1992). In control animals, sham surgery with identical procedure except for ligation of the L5 spinal nerves was received. Any rats exhibiting motor deficiency or lack of tactile allodynia were excluded from the study.

Implantation of intrathecal catheter

Under chloral hydrate (0.3 g/kg, i.p.) anesthesia, implantation of intrathecal cannula was performed following the method of Storkson et al. (Storkson et al., 1996). Briefly, a PE-10 polyethylene catheter was implanted between the L5 and L6 vertebrae to reach the lumbar enlargement of the spinal cord. The outer part of the catheter was plugged and fixed onto the skin on closure of the wound. All surgical procedures were performed under sterile conditions. Rats showing neurological deficits after the catheter implantation were euthanized. Drugs or vehicle were intrathecally injected via the implanted catheter in a 20- μ l volume of solution followed by 10- μ l of normal saline (NS) for flushing. Each injection lasted at least 5 min. After an injection, the needle remained in situ for 2 min before being withdrawn.

Intrathecal delivery of lentivirions: expression and functional detection

Lentivirions including lentivirus-short hairpin SHP2 (LV-shSHP2) and its control LV-GFP were intrathecally delivered at the final titer of 1.0×10^9 TU/ml in 10- μ l volume, respectively. In experiments of knock down SHP2 in naïve rats (Fig. 9), BDNF (100 ng) was intrathecally administrated twice per day, repeated for 2 days on day 5 following lentivirions application. Seven days after BDNF administration and behavioral test, expression of SHP2, SHP1 and GluN2B were detected using Western blot to determine the knockdown of SHP2 by SHP2 siRNA (see Fig. 9I). In experiments of knock down SHP2 in SNL rats (Fig. 10), LV-shSHP2 or LV-GFP was intrathecally delivered at 1.0×10^9 TU/ml in 10- μ l volume on day 5 before SNL operation. Seven days after SNL surgery and behavioral test, expression of SHP2, SHP1 and GluN2B were detected also using Western blot to determine the knockdown of SHP2 by SHP2 siRNA (see Fig. 10G).

Behavioral studies

Assessment of mechanical allodynia

Mechanical allodynia, as a behavioral sign of neuropathic pain, was assessed by measuring 50% paw withdrawal threshold (PWT) as described in our previous reports (Liu et al., 2013; Zheng et al., 2012). The 50% PWT in response to a series of von Frey filaments (Stoelting, Wood Dale, IL) was determined by the Up and Down method (Chaplan et al., 1994). The rat was placed on a metal mesh floor covered with an inverted clear plastic cage (18 × 8 × 8 cm) and allowed a 20-minute period for habituation. Eight von Frey filaments with approximately equal logarithmic incremental (0.224) bending forces were chosen (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g). Each trial started with a von Frey force of 2.00 g delivered perpendicularly to the plantar surface of the left hindpaw for about 2 to 3 seconds. An abrupt withdrawal of the foot during stimulation or immediately after the removal of the hair was recorded as a positive response. Whenever there was a positive or negative response, the next weaker or stronger filament was applied, respectively. This procedure was done until 6 stimuli after the first change in response had been observed. The 50% PWT was calculated using the following formula: $50\% \text{ PWT} = 10^{X_f + k\delta}$, where X_f is the value of the final von Frey filament used (in log units), k is a value measured from the pattern of positive/negative responses, and $\delta = 0.224$, which is the average interval (in log units) between the von Frey filaments (Dixon, 1980). If an animal responded to the lowest von Frey filament, a value of 0.25 g was assigned. If an animal did not respond to the highest von Frey filament, the value was recorded as 15.0 g. In rats, mechanical allodynia is assessed by measuring the 50% PWT to von Frey filaments, and an allodynic rat is defined as that the 50% PWT is <4.0 g (i.e., withdrawal in response to non-noxious tactile stimulus) (Zimmermann, 2001).

Assessment of locomotor function

Inclined-plate test was used for the assessment of locomotor function. Rat was placed crosswise to the long axis of an inclined plate. The initial angle of the inclined plate was 50 degrees. The angle was then adjusted in 5-degree increments. The maximum angle of the plate on which the rat maintained its body position for 5 seconds without falling was determined according to the method reported by Rivlin and Tator (1977). In this study, inclined-plate test was performed for all behavioral experiments which were intrathecally administered drugs or lentivirions to rats.

Measurement of BDNF effects on pain behaviors

The first behavioral experiment was performed to examine whether intrathecal injection of BDNF produced pain hypersensitivity in intact rats (see Fig. 1). It has been shown that the temporal aspects of BDNF application can significantly alter the duration of TrkB activation and its downstream consequences. Acute and gradual increases in BDNF elicit transient and sustained activation of TrkB receptor and its downstream signaling, respectively. In hippocampal slices, fast increase in

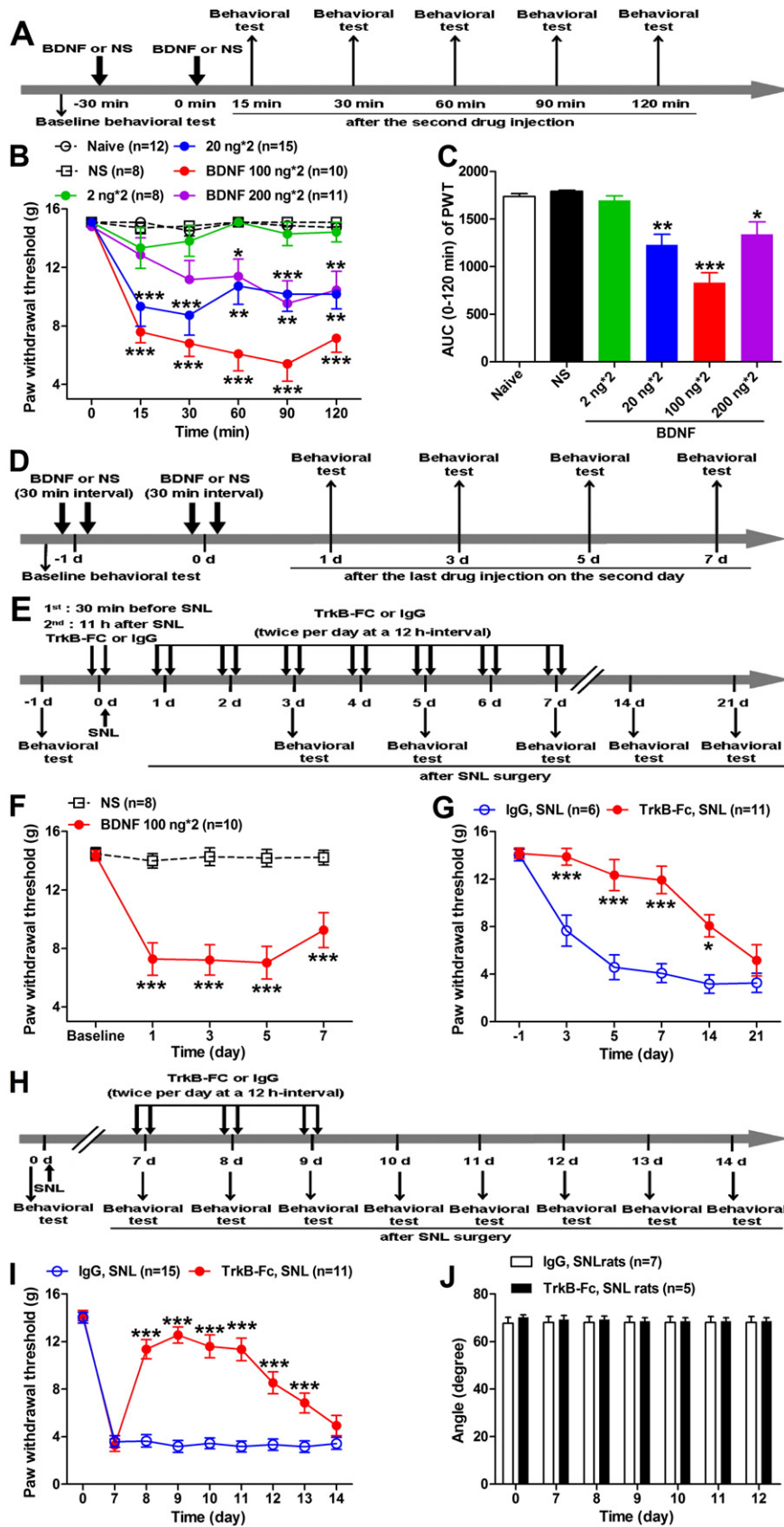
BDNF enhances basal synaptic transmission, whereas slow BDNF delivery facilitates LTP (Ji et al., 2010). On the other hand, endogenous BDNF is increased gradually when the factor is secreted constitutively or from a distant source, although a rapid increase of BDNF can occur locally as a result of regulated secretion triggered by intense neuronal firing (Balkowiec and Katz, 2002; Canossa et al., 1997; Griesbeck et al., 1999; Hartmann et al., 2001; Ji et al., 2010; Lessmann et al., 2003). Thus, in our present study, BDNF at 2, 20, 100 and 200 ng in 20- μ l volume, or vehicle (NS) in an equal volume, was intrathecally administered to animals, repeated twice at a 30 min-interval of each dose according to the methods described in previous reports (Geng et al., 2010; Ji et al., 2010). 50% PWT was measured just before drug injection, and then measured at 15, 30, 60, 90 and 120 min after the second drug injection, respectively (see Fig. 1A). To examine whether BDNF produced a long-lasting effect on mechanical allodynia, BDNF at 100 ng was intrathecally administered twice per day at a 30 min-interval, continued for 2 days (i.e. there were two times two injections at a 30 min-interval per day on two consecutive days), and the 50% PWT was measured on the day before and then on day 1, 3, 5 and 7 after the last drug injection on the second day, respectively (see Fig. 1D).

The second behavioral experiment was carried out to determine whether pre-treatment or post-treatment with TrkB-Fc, a TrkB-immunoglobulin G fusion protein that is usually applied to scavenge endogenous BDNF, would alleviate pain hypersensitivity in neuropathic rats (see Figs. 1E and G–J). In pre-treatment experiment, TrkB-Fc at 0.5 μ g/ μ l (10 μ g in 20- μ l volume), or IgG at an equal dose, was intrathecally delivered to rats 30 min prior to SNL surgery, again at the end of the first day (~11 h after surgery), and then repeated twice per day (at a 12 h-interval) in the following 7 days after SNL surgery. 50% PWT to von Frey filaments was then measured before SNL and on day 3, 5, 7, 14 and 21 after SNL surgery, respectively (see Fig. 1E). In post-treatment experiment, on day 7 after SNL operation, TrkB-Fc at 0.5 μ g/ μ l (or IgG at an equal dose), was intrathecally administered to rats exhibiting mechanical allodynia twice per day (at a 12 h-interval) continued for 3 days. The 50% PWT was measured before SNL and repeated from day 7 to day 14 after SNL surgery, respectively (see Fig. 1H).

Measurement of SHP2 effects on pain behaviors

Firstly, we examined whether the BDNF-induced mechanical allodynia in intact rats could be inhibited by spinal application of NSC-87877 (a potent SHP2 protein tyrosine phosphatase inhibitor) (see Fig. 7) or by knock-down of SHP2 in the spinal cord using SHP2 siRNA (see Fig. 9). BDNF (at 100 ng) was intrathecally applied twice per day for 2 days as described above (i.e. there were two time two injections at a 30 min-interval per day on two consecutive days), and then NSC-87877 at 2.5 ng/ μ l (50 ng in 20- μ l volume), or vehicle (DMSO) in an equal volume, was intrathecally administered to rats on day 3 after the last BDNF injection. The 50% PWT of rat was measured before and at 1, 3 and 5 h after NSC-87877 application, respectively (see Fig. 7I). In order to knock down spinal SHP2 in intact rats, lentivirus-short hairpin SHP2 (LV-shSHP2) or its control LV-GFP were intrathecally

Fig. 1. Intrathecal administration of exogenous BDNF mimics pain allodynia in intact rats, while spinal application of TrkB-Fc attenuates pain hypersensitivity in SNL rats. (A–C) Effects of BDNF on pain behaviors of intact rats. (A) Scheme of the experimental procedure. BDNF (or NS) was intrathecally administered to animals, repeated twice at a 30 min-interval. The paw withdrawal threshold (PWT) was measured just before drug injection, and then measured at 15, 30, 60, 90 and 120 min after the second drug injection, respectively. Note that BDNF (at 20, 100 and 200 ng) statistically reduces the paw withdrawal threshold (PWT) of rat in an incomplete dose-dependent manner (B). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to NS, two-way ANOVA, $n = 8–15$ /group. (C) Area under the time-course curve (AUC) of PTW obtained from (B). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to NS, one-way ANOVA, $n = 8–15$ /group. (D and F) Long-lasting effects of BDNF on pain behaviors of intact rats. (D) Scheme of the experimental procedure. BDNF (100 ng) was intrathecally administered to rats twice per day at a 30 min-interval, repeated for 2 days, and the PWT was measured on the day before drug and then on day 1, 3, 5 and 7 after the last drug injection on the second day, respectively. Note that repeated administration of BDNF (100 ng, twice per day at a 30 min-interval, continued for 2 days) produces a long-time decrease in PWT of rat that lasted at least for 7 days after drug injection (F). *** $P < 0.001$, compared to NS, two-way ANOVA, $n = 10$ BDNF and 8 NS. (E and G to J) Effects of TrkB-Fc on mechanical allodynia and locomotor function in SNL rats. (E and H) Scheme of the experimental procedure for pre-treatment (E) and post-treatment experiment (H), respectively. In pre-treatment experiment, TrkB-Fc (0.5 μ g/ μ l) was intrathecally delivered to rats 30 min prior to SNL surgery, again at the end of the first day, and then repeated twice per day (at a 12 h-interval) in the following 7 days after SNL surgery. The PWT was measured before SNL and on day 3, 5, 7, 14 and 21 after SNL surgery, respectively (E). In post-treatment experiment, on day 7 after SNL operation, TrkB-Fc was intrathecally administered to rats exhibiting mechanical allodynia twice per day (at a 12 h-interval) continued for 3 days. The PWT was measured before SNL and repeated from day 7 to day 14 after SNL surgery, respectively (H). Note that either pre-treatment or post-treatment with TrkB-Fc can inhibit the nerve injury-induced decrease in PWT of SNL rats. * $P < 0.05$, *** $P < 0.001$, compared to control IgG, two-way ANOVA, $n = 11$ TrkB-Fc, 6 IgG (pre-treatment) and 11 TrkB-Fc, 15 IgG (post-treatment), respectively. (J) Intrathecal injection of TrkB-Fc had no significant damage to the locomotor function of rats ($p > 0.05$, two-way ANOVA, $n = 5$ TrkB-Fc and 7 IgG).



delivered at the final titer of 1.0×10^9 TU/ml in 10- μ l volume. Five days after lentivirions injection, BDNF (at 100 ng) was intrathecally administered twice per day at a 30 min interval, continued for 2 days. Both PWT and locomotor function were measured before lentivirions injection, and then on day 1, 3, 5 and 7 after the last BDNF administration, respectively (see Fig. 9I).

Secondly, we investigated whether inhibition or knockdown of spinal SHP2 would alleviate pain hypersensitivity in neuropathic rats. On day 7 after SNL operation, NSC-87877 (2.5 ng/ μ l), or vehicle (DMSO) in an equal volume, was intrathecally delivered to rats exhibiting mechanical allodynia. The 50% PWT was measured before SNL surgery and NSC-87877 injection, and then at 1, 3 and 5 h after NSC-87877 application, respectively (see Fig. 8I). In SHP2 knockdown experiments, LV-shSHP2 or LV-GFP was intrathecally delivered at 1.0×10^9 TU/ml in 10- μ l volume on day 5 before SNL surgery. Both PWT and locomotor function were measured before lentivirions injection and SNL operation, and then on day 1, 3, 5 and 7 after SNL surgery, respectively (see Fig. 10G).

Western blot

Synaptosomal fraction of rat lumbar spinal dorsal horn was extracted using modified methods as previously described (Dunah and Standaert, 2001). Briefly, under deep anesthesia with 10% chloral hydrate (0.3 g/kg, i.p.), the lumbar spinal dorsal horn around L4–L5 segment was removed and immediately homogenized in ice-chilled lysis buffer containing 320 mM sucrose, 10 mM HEPES, 2 mM EDTA and 1 mM PMSF. The extract was centrifuged at 1000 \times g for 10 min to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged at 10,000 \times g for 15 min to obtain the synaptosomal fraction (P2). The P2 pellet were solubilized with 1% SDS in TEVP buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF and 1% SDS at 4 °C for 1 h, and centrifuged at 15,000 \times g for 5 min to get the supernatant. The concentration of protein was measured with a BCA assay kit (Pierce, Rockford, IL), and equal amount of protein samples (60 μ g) were denatured and then separated through sodium dodecyl sulphate-polyacrylamide (SDS) gel electrophoresis using 8% running gels and transferred to a polyvinylidene difluoride filters (PVDF) membrane (Millipore). After blocking with 3% BSA in Tris-buffered saline and Tween (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween-20) for 60 min at room temperature, the membranes were respectively incubated with the following primary antibodies at 4 °C overnight: mouse anti-SHP2 (1:1000, BD Transduction Laboratories), rabbit anti-pSHP2 (1:1000, Cell signaling), mouse anti-GluN2B (1:1000, Cell signaling), or mouse anti-GAPDH (1:1000, Santa Cruz Biotechnology). The blots were washed in Tris-buffered saline and Tween and then incubated in horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG secondary antibody (1:1000, Santa Cruz Biotechnology). Protein bands were visualized using an enhanced chemiluminescence detection kit (Pierce) followed by autoradiography using Hyperfilm MP (Santa Cruz Biotechnology). The standardized ratio of SHP2 or GluN2B to GAPDH band density was used to calculate the alteration of SHP2 or GluN2B expression. The ratio of phosphorylated SHP2 to total SHP2 was performed to assess the activated SHP2.

Electrophysiological studies

Surgery

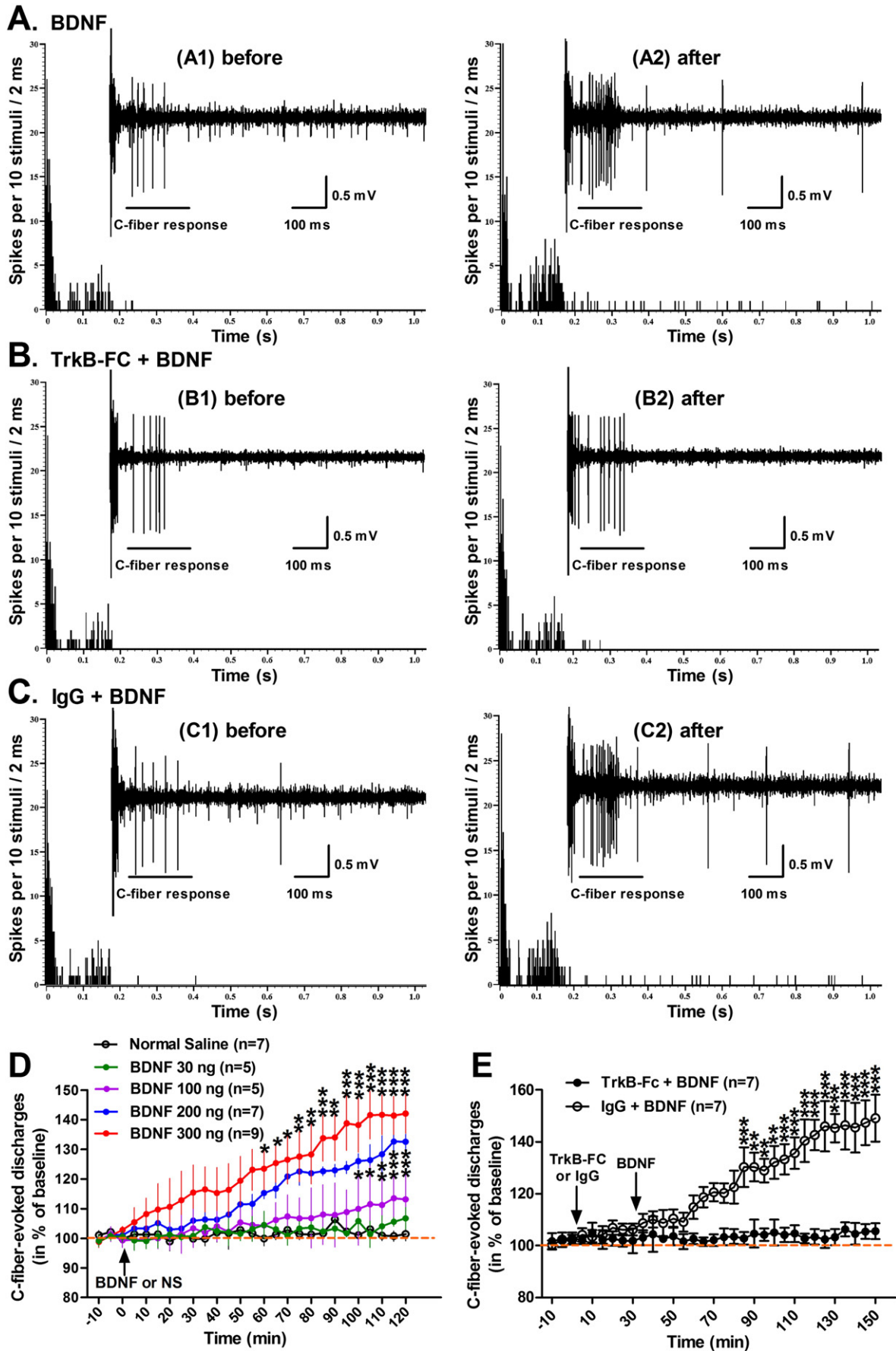
The rat was initially anesthetized with urethane (1.2–1.5 g/kg, i.p.). The trachea was cannulated to allow mechanical ventilation with room air. A catheter was inserted into the right jugular vein for continuous infusion of Tyrode's solution containing (in mM) NaCl 137, KCl 2.7, CaCl₂ 1.4, MgCl₂ 1.0, NaHCO₃ 6.0, NaH₂PO₄ 2.1, D-(+)-glucose 6.5, pH 7.4, at a rate of 1.5–2 ml/h. The rectal temperature was maintained at 36.5–37.5 °C via a feedback-controlled under-body heating pad. A pair of bipolar silver hook electrode was placed under the sciatic nerve immediately proximal to the trifurcation for electrical stimulation. The vertebral column was rigidly fixed in the frame with two clamps. The lumbar enlargement of the spinal cord was exposed by laminectomy at the vertebrae T13 and L1 and the dura covering lumbosacral spinal segments was carefully removed. A small well was built with 3% agar on the dorsal spinal cord at the recording segment to allow application of drugs or vehicles as described in our previous reports (Liu et al., 2010; Qu et al., 2009). The exposed spinal tissue was covered with warm (37 °C) saline solution.

After surgery, the animal was artificially ventilated with a small animal ventilator and paralyzed with curare (2.0 mg/kg, i.v.), and continuous anesthesia and paralysis were maintained with urethane (0.10–0.15 g/kg/h) and curare (0.20 mg/kg/h) during the whole experiment. The depth of anesthesia was monitored by examination of papillary size and reflexes. The physiological condition of the animal was monitored by recording the electrocardiogram (330–460 beats/min), end-expiratory CO₂ (3.5–4.5%), and rectal temperature (36.5–37.5 °C), and was maintained within the range indicated. All the drugs were applied topically to the dorsal surface of the spinal cord in a 20- μ l volume of solution.

Wide dynamic range (WDR) neuron recording

Single-unit extracellular recording was made from the lumbar dorsal horn neuron within 1200 μ m of the dorsal surface in the spinal cord with 2–5 M Ω parylene-coated tungsten microelectrodes (Friedrick Haer & Co., Bowdoinham, ME, USA), because the WDR neurons are located in the deep dorsal horn, mainly in laminae IV and V of the dorsal horn in the spinal cord. The microelectrode was inserted perpendicularly into the dorsal horn from a point about midway between the midline and the medial edge of the dorsal root entry zone. During electrode advancement, electrical pulses (0.3-ms duration, 0.4 mA, 0.5 Hz) were applied to the ipsilateral sciatic nerve as search stimuli so that a neuron with no spontaneous firing could be identified. Once a single unit was identified, the receptive field and response characteristics were determined by a range of mechanical stimuli of varying intensities, including brushing or touching the skin with a cotton brush, light pressure with a probe, and pinching a fold of skin with toothed forceps. A neuron responding to innocuous tactile stimuli, light pressure, and noxious pinch in a graded manner was identified as a wide dynamic range (WDR) neuron and was selected for further investigation (Liu et al., 2010; Qu et al., 2009). The recorded signals were amplified with an AC pre-amplifier, filtered with a passing band width 500–1000 Hz, displayed on an oscilloscope, and fed to a Pentium computer via a CED

Fig. 2. Spinal application of BDNF induces a long-lasting increase in the C-fiber responses of dorsal horn WDR neurons in intact rats. (A–C) Examples showing effects of BDNF on the C-fiber responses of dorsal horn WDR neurons (A) and effects of TrkB-FC (B) or control IgG (C) on BDNF-induced activities of WDR neurons. Panels illustrate the post-stimulus histogram of the electrically evoked neuronal responses in a dorsal horn WDR neuron 30 min before (A1 to C1) and 120 min after (A2 to C2) BDNF (300 ng) application, respectively. Inset shows the original recordings of the first electrically evoked neuronal responses corresponding to each time point respectively. (D) Statistical analysis of BDNF (30, 100, 200 and 300 ng) on the C-fiber responses of WDR neurons. Note that at both 200 and 300 ng, BDNF significantly enhances the C-fiber responses of WDR neurons, and these potentiated effects of BDNF last at least for 120 min after drug administration. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to NS, two-way ANOVA, $n = 5–9$ /group. (E) Statistical analysis of TrkB-FC or IgG on BDNF-induced activities of WDR neurons. Note that the BDNF-induced increase in C-fiber responses of WDR neurons is almost completely blocked by spinal application of TrkB-Fc (0.5 μ g/ μ l) 30 min before BDNF (300 ng) administration. ** $P < 0.01$, *** $P < 0.001$, compared to IgG, two-way ANOVA, $n = 7$ /group.



1401 interface for off-line analysis using the Spike2 software (Cambridge Electronic Design, Cambridge, UK). Spikes appearing 45–300 ms after stimulus were defined as C-fiber responses, i.e. responses in the WDR neurons evoked by C-fiber activation (Liu et al., 2010; Qu et al., 2009). Single-cell recording was ensured on the basis of amplitude and shape of the action potentials. Then, a train of 10 stimuli (0.5-ms duration, 0.5 Hz, with a pulse current of $2 \times$ C-fiber response threshold), which was used as test stimulus, was applied repeatedly to the sciatic nerve at 5-min intervals, and post-stimulus histograms from the responses of WDR neurons were generated by the Spike2 software. In this study, only C-fiber responses of WDR neurons, which are highly related to nociceptive transmission, were examined and analyzed. All of the C-fiber responses values are expressed as percentages of the mean response value of three pre-drug consecutive trains of test stimuli. Cells showing variation of less than 20% were selected for further experiments. In the following electrophysiological studies, only one cell was studied in each animal, and each animal received only one dose of drugs.

Measurement of BDNF effects on WDR neurons

Firstly, we tested effects of spinal application of BDNF on C-fiber responses of dorsal horn WDR neuron in intact rats. After three stable control responses were recorded, BDNF at 30, 100, 200 and 300 ng in 20- μ l volume, or an equal volume of vehicle (NS), was applied topically to the dorsal surface of the spinal cord respectively, and the post-drug responses evoked by the same test stimulus as described above were measured at 5-min intervals for up to 120 min (see Fig. 2D). Secondly, we explored whether the BDNF-induced activities of WDR neurons could be blocked by TrkB-Fc. Similarly, after three stable control responses were recorded, TrkB-Fc or control IgG at 0.5 μ g/ μ l (10 μ g in 20- μ l volume) was spinally administrated 30 min before BDNF (300 ng) application, and the post-drug responses were measured for up to 120 min after BDNF administration (see Fig. 2E).

Field potential recording

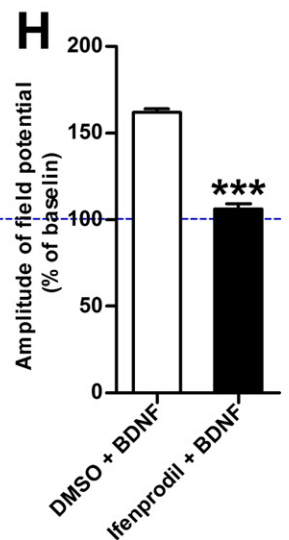
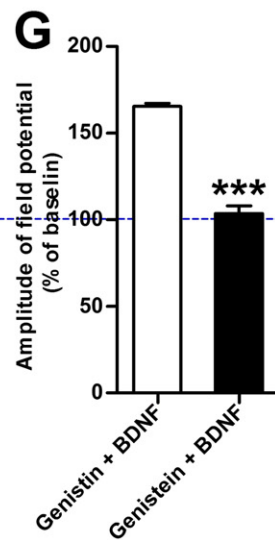
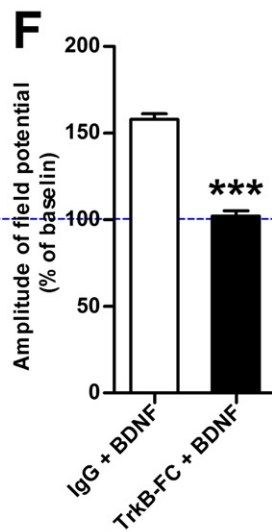
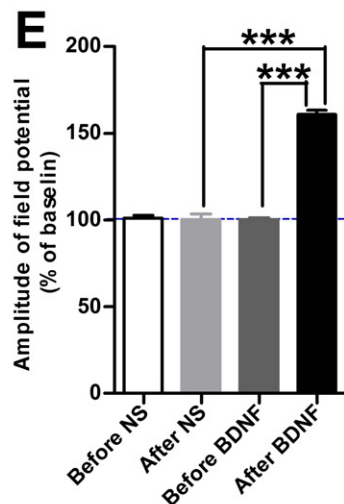
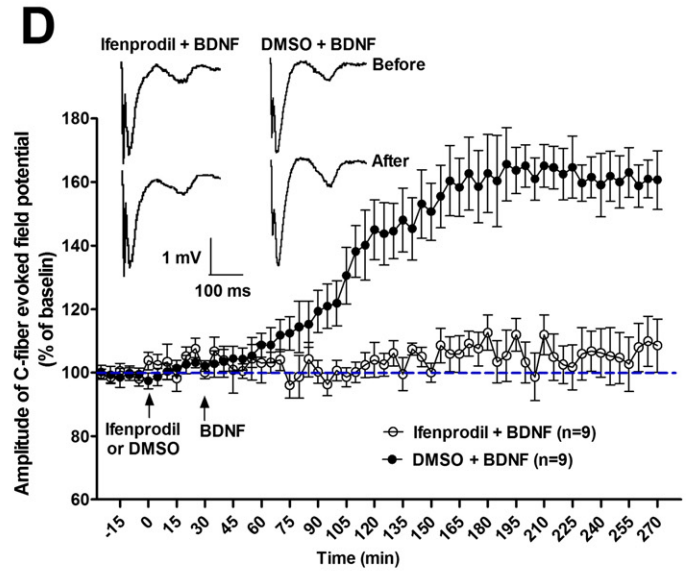
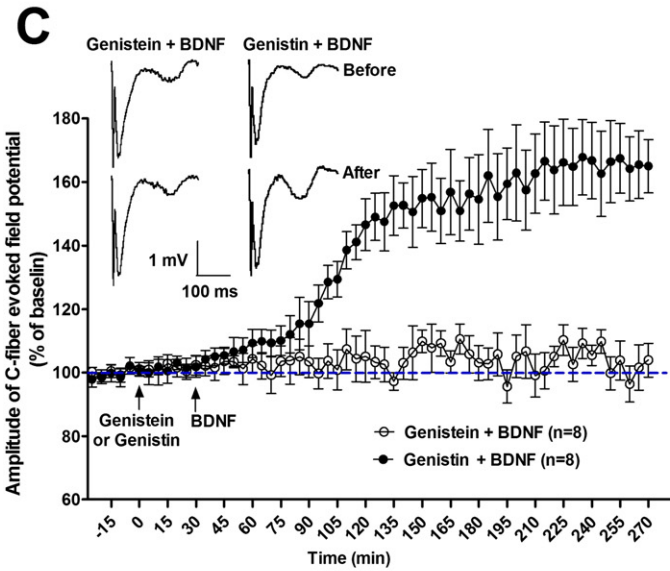
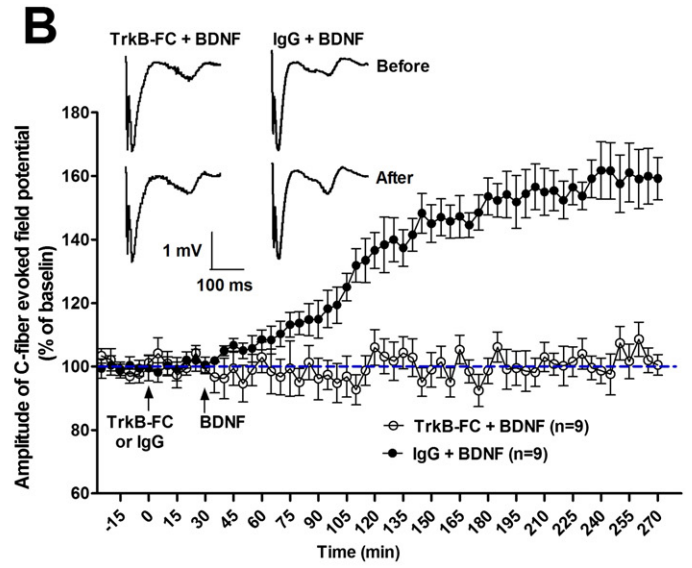
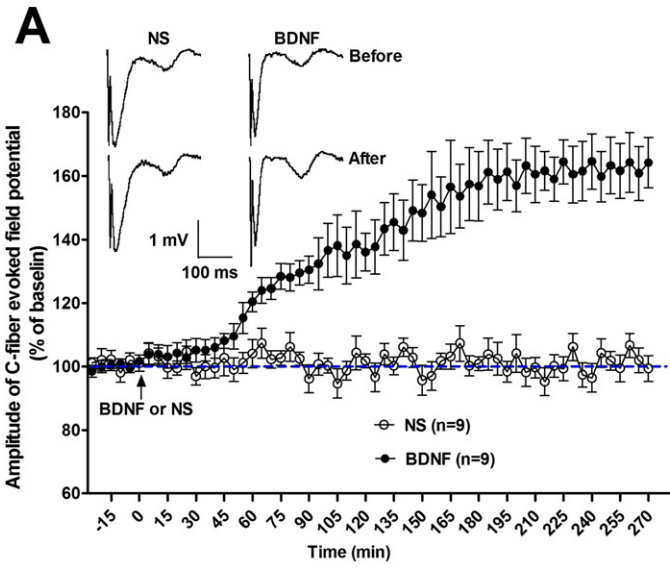
The C-fiber-evoked field potentials were recorded at a depth of 100–50 μ m from the dorsal surface of L4–L5 spinal cord with 1–3 M Ω parylene-coated tungsten micro-electrodes (Friedrick Haer & Co., Bowdoinham, ME, USA), driven by a micro-stepping motor. A bandwidth of 0.1–300 Hz was used to remove artifacts without altering the C-fiber-evoked field potentials. The signals were amplified, filtered and displayed on an oscilloscope, and fed to a Pentium computer via a CED 1401 interface for off-line analysis using the Spike2 software (Cambridge Electronic Design, Cambridge, UK). A test stimulation of a single square pulse (0.5-ms duration, delivered at 2-s intervals) was applied to the sciatic nerve for measuring the threshold of the evoked field potentials. The intensity of the stimulation was increased gradually from 0 V to the voltage intensity just evoking the C-fiber-evoked field potentials as described in our previous study (Xing et al., 2007). The intensity of the stimulation that would just elicit the C-fiber-evoked field potentials was defined as the threshold of the evoked field potentials. Following this measurement, another test stimulation of a single square pulse (0.5-ms duration, 10–20 V, delivered at 5-min intervals) was applied to the sciatic nerve to evoke spinal C-fiber field potentials for at least 30 min as baseline control. The mean amplitude of the control field potentials was obtained from an average of 6 individual test potentials (100%).

LTP induction

For BDNF-induced LTP: after six stable control field potentials were recorded, BDNF at 300 ng (in 20- μ l volume), or an equal volume of vehicle (normal saline), was then applied topically to the dorsal surface of the spinal cord, and the post-drug field potentials evoked by the same test stimulus (0.5-ms duration, 10–20 V, delivered at 5-min intervals) as described above were measured at 5-min intervals for up to 270 min (see Fig. 3A). To test the pharmacological effect of some inhibitors (e.g. the tyrosine kinase inhibitor TrkB-Fc (see Fig. 3B) or genistein (see Fig. 3C), the selective GluN2B antagonist ifenprodil (see Fig. 3D), and the SHP2 antagonist NSC-87877 (see Fig. 7F) on BDNF-induced LTP in intact rats, an inhibitor or its inactive analogue (or vehicle) was spinally administrated 30 min before BDNF application. The mean amplitude of the control field potentials (the baseline responses) was obtained from an average of 6 individual test potentials (100%), and the amplitude of the field potential evoked by each test-stimulation after administration of BDNF (or inhibitor) was normalized and expressed as the percentage of the baseline responses. Eight to nine animals were used in each group for these experiments. Moreover, to investigate effects of SHP2 knockdown on BDNF-induced LTP in intact rats (see Fig. 9E), LV-shSHP2 or control LV-GFP was intrathecally delivered to rats at 1.0×10^9 TU/ml in 10- μ l volume, and then the BDNF-induced LTP were recorded 7 days after lentivirions injection and behavioral test in the same manner as described above (see Fig. 9I). Five animals were used in each group for these experiments.

For high-frequency electrical stimulation (HFS)-induced LTP: after six stable control field potentials were recorded, a high-frequency conditioning stimulation (0.5-ms duration, 30–40 V, 100 Hz, 400 pulses given in 4 trains of 1-s duration at 10-s intervals) was then delivered to the sciatic nerve, and the post-HFS field potentials evoked by the same test stimulus (0.5-ms duration, 10–20 V, delivered at 5-min intervals) as described above were measured at 5-min intervals for up to 240 min (see Fig. 4A). The mean amplitude of the control field potentials (the baseline responses) was obtained from an average of 6 individual test potentials (100%), and the amplitude of the field potential evoked by each test-stimulation after HFS was normalized and expressed as the percentage of the baseline responses. To test effects of pre-treatment with TrkB-Fc (see Fig. 4B) or ifenprodil (see Fig. 4C) on HFS-induced LTP in SNL rats, TrkB-Fc (0.5 μ g/ μ l) or ifenprodil (1.0 μ g/ μ l) was intrathecally delivered to rats 30 min prior to SNL operation, again at the end of the first day (~11 h after surgery), and then repeated twice per day (at a 12 h interval) in the following 7 days after SNL surgery. An equal dose of IgG or DMSO was delivered to rats as control in the same procedure. Electrophysiological recording was performed 7 days after SNL surgery and behavioral test (see Fig. 4E). To explore effects of NSC-87877, a SHP2 inhibitor, on HFS-induced LTP in SNL rats, NSC-87877 (2.5 μ g/ μ l) or vehicle DMSO at an equal dose was spinally applied to the surface of spinal dorsal horn 30 min after the baseline responses recording. Sixty minutes later, a HFS (0.5-ms duration, 30–40 V, 100 Hz, 400 pulses given in 4 trains of 1-s duration at 10-s intervals) was delivered to the sciatic nerve, and the post-HFS field potentials evoked by the same test stimulus as described above were measured at 5-min intervals for up to 240 min (see Fig. 8F). In addition, to examine effects of SHP2 knockdown on HFS-induced LTP in SNL rats (see Fig. 10E), LV-shSHP2 or LV-GFP was intrathecally delivered to rats as described in aforementioned methods (i.e., at 1.0×10^9 TU/ml in 10- μ l volume on day 5 before SNL operation), and LTP was induced by a HFS (0.5-ms

Fig. 3. Spinal application of BDNF induces LTP of C-fiber-evoked field potentials in the spinal dorsal horn via activation of GluN2B-NMDA receptors. (A) Effects of spinal BDNF on amplitude of C-fiber-evoked field potentials. Note that spinal application of BDNF (300 ng) induces a long latency LTP of C-fiber-evoked field potentials in the spinal dorsal horn in intact rats. Traces at top are recorded at 10 min before and 3 h after drug (BDNF or NS) application, respectively. (B–D) Effects of TrkB-Fc, genistein or ifenprodil on BDNF-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn. Note that perfusion of either TrkB-Fc (0.5 μ g/ μ l) (B), genistein (5 μ g/ μ l) (C) or ifenprodil (1 μ g/ μ l) (D) for 30 min before spinal application of BDNF (300 ng) can almost completely block the BDNF-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn. Traces at top are recorded at 10 min before and 3 h after BDNF application, respectively. (E) Statistical analysis of mean C-fiber-evoked field potentials during 30 min before and 3–4 h after drug (BDNF or NS) application, respectively. **** $P < 0.001$, one-way ANOVA, $n = 8$ /group. (F–H) Comparison of mean C-fiber-evoked field potentials during 3–4 h after BDNF application between the TrkB-Fc and the control IgG group (F), the genistein and its inactive analogue of genistein group (G) as well as the ifenprodil and the vehicle DMSO group (H), respectively. **** $P < 0.001$, two-tailed unpaired t-test, $n = 8$ –9/group. TrkB-Fc, a TrkB-immunoglobulin G fusion protein, is applied to scavenge endogenous BDNF; genistein, a tyrosine kinase inhibitor; ifenprodil, a selective GluN2B-NMDA receptor inhibitor.



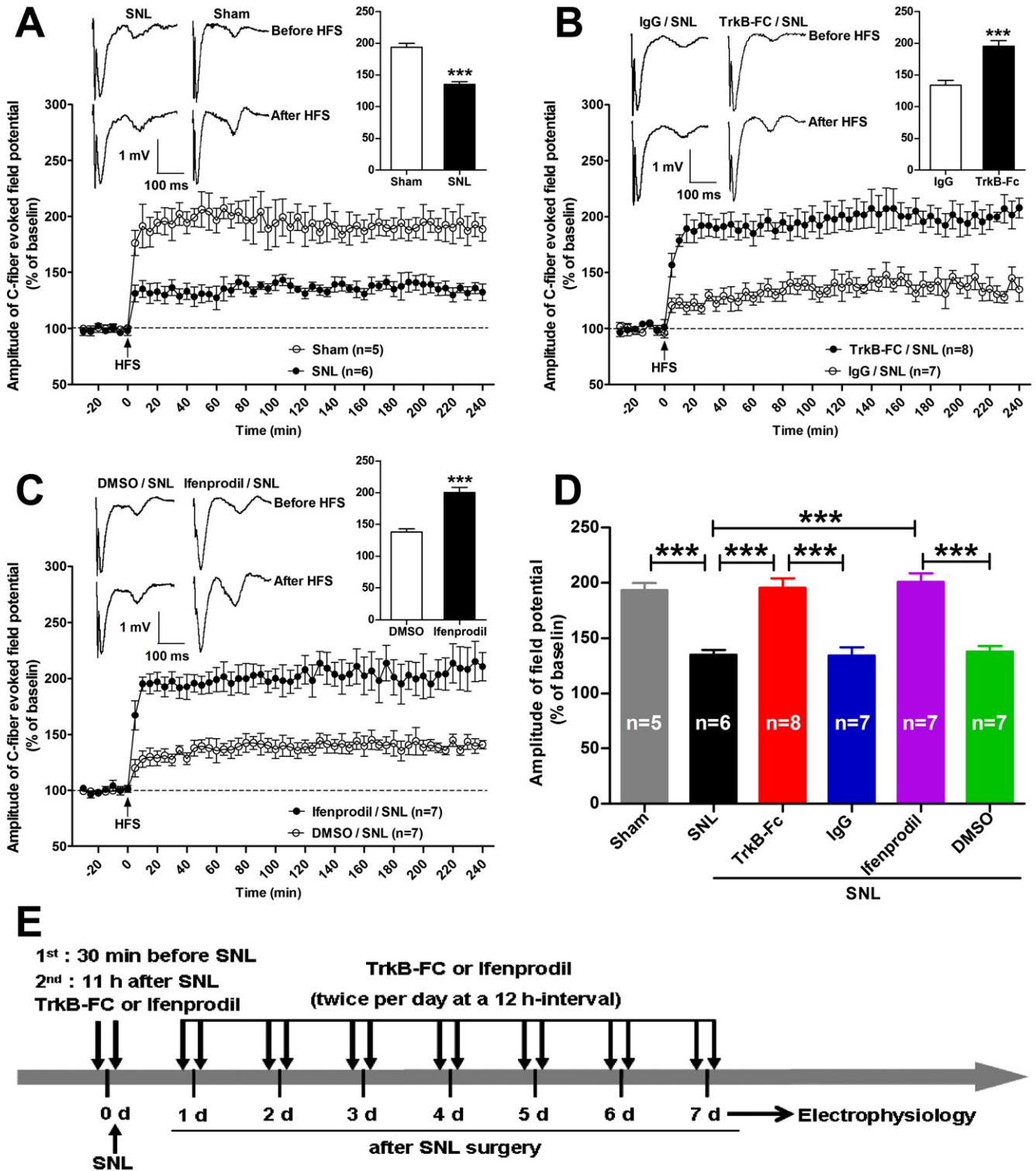


Fig. 4. Pre-treatment with TrkB-Fc or ifenprodil inhibits the decrease of spinal LTP induced by high-frequency electrical stimulation (HFS) of the sciatic nerve in SNL rats. Field potentials in the spinal dorsal horn are elicited by the test electrical stimulus (0.5-ms duration, 10–20 V) of the sciatic nerve at 5-min intervals. (A) Mean time course of C-fiber-evoked field potentials before and after HFS (0.5-ms duration, 30–40 V, 100 Hz, 400 pulses given in 4 trains of 1-s duration at 10-s intervals; arrow) in SNL (filled circles, $n = 6$) and sham-operated rats (open circles, $n = 5$). Traces at top are recorded at 10 min before and 3 h after HFS of the sciatic nerve in SNL and sham-operated animals, respectively. The bar graph indicates the mean amplitude of C-fiber-evoked field potentials during the 3–4 h following HFS in SNL and sham-operated animals. Note that the HFS-induced LTP in the spinal dorsal horn is markedly occluded in SNL rats compared to sham-operated rats. $***P < 0.001$, two-tailed unpaired *t*-test. (B and C) Effects of pre-treatment with TrkB-Fc or ifenprodil on spinal LTP of C-fiber-evoked field potentials induced by HFS of the sciatic nerve in SNL rats. Note that pre-treatment with TrkB-Fc (B) or ifenprodil (C) prominently inhibit the decrease of HFS-induced LTP of C-fiber-evoked field potentials in SNL rats. Traces at top are recorded at 10 min before and 3 h after HFS of the sciatic nerve in TrkB-Fc and IgG pretreated animals (B) as well as in ifenprodil and DMSO pretreated animals (C), respectively. The bar graph indicates the mean amplitude of C-fiber-evoked field potentials during the 3–4 h following HFS in each corresponding group. $***P < 0.001$, two-tailed unpaired *t*-test, $n = 7$ –8/group. (D) Statistical analysis of mean C-fiber-evoked field potentials during the 3–4 h following HFS of the sciatic nerve in rats obtained from each above group. $***P < 0.001$, one-way ANOVA, $n = 5$ –8/group. (E) Scheme of the experimental procedure. TrkB-Fc (0.5 $\mu\text{g}/\mu\text{l}$) or ifenprodil (1.0 $\mu\text{g}/\mu\text{l}$) was intrathecally delivered to rats 30 min prior to SNL operation, again at the end of the first day, and then repeated twice per day (at a 12 h-interval) in the following 7 days after SNL surgery. Electrophysiological recording was performed 7 days after SNL surgery and behavioral test.

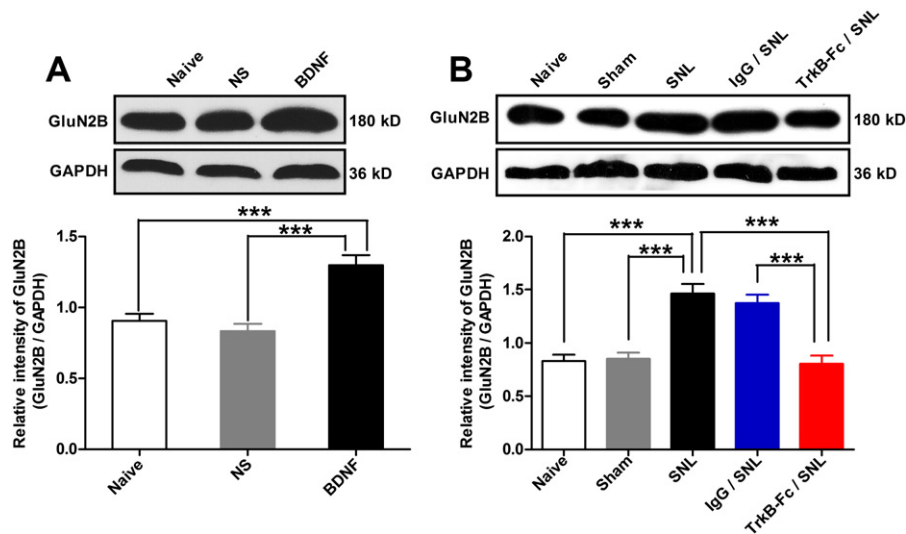


Fig. 5. Intrathecal injection of BDNF enhances the expression of dorsal horn GluN2B in intact rats, while pre-treatment with TrkB-Fc prevents the nerve injury-induced up-regulation of GluN2B in SNL rats. (A) Western blot of GluN2B expression in synaptosomal fraction extracted from the spinal dorsal horn tissue in naive, NS-treated, and BDNF-treated rats. Upper: representative of Western blot bands; lower: analysis of the relative intensity of GluN2B. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as internal control. Note that the expression of GluN2B is markedly increased in BDNF-treated rats as compared to naive and NS-treated rats, respectively. $***P < 0.001$, one-way ANOVA, $n = 5$ /group. (B) Western blot of GluN2B expression in synaptosomal fraction extracted from the spinal dorsal horn tissue in naive, SNL, sham-operated rats, and in SNL rats pre-treated with TrkB-Fc or IgG. Upper: representative of Western blot bands; lower: analysis of the relative intensity of GluN2B. Note that the expression of GluN2B is prominently increased in SNL rats as compared to naive and sham-operated rats, respectively. Moreover, the SNL-induced up-regulation of GluN2B expression is almost completely rescued by preemptive application of TrkB-Fc. $***P < 0.001$, one-way ANOVA, $n = 6$ /group.

duration, 30–40 V, 100 Hz, 400 pulses given in 4 trains of 1-s duration at 10-s intervals) to the sciatic nerve of neuropathic rats 7 days after SNL surgery and behavioral test (see Fig. 10G). Five to eight animals were used in each group for these experiments.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA). All data were expressed as mean \pm SEM. A two-tailed unpaired t-test was used for the comparison of the mean values between two groups. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or two-way ANOVA followed by the Bonferroni post-hoc test was used for multiple comparison. Area under the time-course curve (AUC) values during the analysis time was used to measure the summarized effects of different treatment or at different doses (Qu et al., 2009). Differences with $P < 0.05$ were considered statistically significant.

Results

Spinal BDNF contributes to the pathogenesis of neuropathic pain

In a previous study, we have reported that BDNF/TrkB-mediated signaling pathway in the spinal cord is involved in the development of neuropathic pain through activation of dorsal horn GluN2B-NMDA receptors after peripheral nerve injury (Geng et al., 2010). In this study, we first confirmed the contribution of spinal BDNF to the pathogenesis of neuropathic pain by showing that intrathecal (i.t.) administration of BDNF (Fig. 1A) could mimic significant mechanical allodynia in intact rats in an incomplete dose-dependent manner (Figs. 1B and C). We found that at both 20 ng*2 and 100 ng*2, BDNF produced a significant decrease in paw withdrawal threshold (PWT) of rats from 15 min after the last drug injection, lasted for 120 min until experiment termination. However, as dose increased to 200 ng*2, the effect of BDNF on the PWT reduced and the beginning of drug efficacy also delayed to 60 min post-drug application ($p < 0.05$ to 0.001, compared to control NS, Fig. 1B). When summarized in area under the time-course curve (AUC) of PTW, the AUC (0–120 min of the analysis time)

was significantly decreased to 1230 ± 109.6 ($p < 0.05$), 832.2 ± 104.3 ($p < 0.001$) and 1341 ± 129.8 ($p < 0.01$) from 1791 ± 10.4 of control NS after BDNF injection at 20 ng*2, 100 ng*2 and 200 ng*2, respectively (Fig. 1C). As assessed by inclined-plate test, no significant motor dysfunction was found in rats after intrathecal administration of BDNF at maximal dose of both 100 ng*2 and 200 ng*2 (data not shown).

To examine long-lasting effects of spinal BDNF on pain behaviors, BDNF at 100 ng was intrathecally administrated to rat twice per day at a 30 min-interval, continued for 2 days, and the effect of BDNF on PWT was tested from day 1 to day 7 after the last drug injection on the second day (Fig. 1D). The results showed that repeated administration of BDNF for two days produced a long-time mechanical allodynia at least for 7 days after drug injection. As shown in Fig. 1F, the PWT (in gram) was markedly decreased from day 1 (7.27 ± 1.1 BDNF vs. 13.99 ± 0.5 NS, $p < 0.001$) to day 7 (9.25 ± 1.2 BDNF vs. 14.22 ± 0.5 NS, $p < 0.001$) after BDNF injection. Similarly, the results of inclined-plate test showed that repeated administration of BDNF for two days had no obvious motor dysfunction to rats at any time point of post-drug injection (data not shown).

To further confirm the role of endogenous BDNF in pathogenesis of neuropathic pain, we first examined whether pre-treatment with TrkB-Fc to scavenge endogenous BDNF would prevent or alleviate pain hypersensitivity in neuropathic rats. We observed that pre-treatment with TrkB-Fc (0.5 μ g/ μ l) prior to SNL surgery (Fig. 1E) significantly inhibited SNL-induced decrease in PWT (in gram) from day 3 (13.88 ± 0.7 TrkB-Fc vs. 7.66 ± 1.3 IgG, $p < 0.001$) to day 14 (8.08 ± 0.9 TrkB-Fc vs. 3.16 ± 0.8 IgG, $p < 0.05$) following spinal nerve injury (Fig. 1G), indicating that pre-treatment with TrkB-Fc alleviates the nerve injury-induced mechanical allodynia in SNL rats. Next, we determined whether post-treatment with TrkB-Fc after SNL surgery could rescue the nerve injury-induced pain allodynia in neuropathic rats. Similarly, post-treatment with TrkB-Fc (see Fig. 1H) could also dramatically inhibit the SNL-induced decrease in PWT (in gram) from day 8 (11.37 ± 0.8 TrkB-Fc vs. 3.65 ± 0.5 IgG, $p < 0.001$) to day 13 (6.84 ± 0.8 TrkB-Fc vs. 3.17 ± 0.5 IgG, $p < 0.05$) after nerve injury (Fig. 1I), implying that post-treatment with TrkB-Fc to scavenge endogenous BDNF in the spinal cord can partly rescue the nerve injury-induced mechanical allodynia in neuropathic rats. Moreover, we performed

inclined-plate test and found that either intrathecal administration of TrkB-Fc or IgG at our experimental dosage had no significant damage to the locomotor function of rats ($p > 0.05$, Fig. 1J).

BDNF induces long-lasting hyperexcitability of WDR neurons in the spinal dorsal horn

To further elucidate the potential mechanisms by which how spinal BDNF contributes to the pathogenesis of neuropathic pain, we first examined effects of BDNF on C-fiber responses of dorsal horn WDR neurons, which are highly related to nociceptive transmission (Qu et al., 2009; Rygh et al., 2000). Our results showed that spinal application of BDNF induced long-lasting hyperexcitability of dorsal horn WDR neurons as assessed by measuring the C-fiber evoked discharges (Fig. 2). We found that at either 200 ng or 300 ng, BDNF significantly increased the C-fiber responses of WDR neurons, and these potentiated effects of BDNF lasted at least for 120 min after drug administration. The C-fiber responses was increased prominently from $101.47 \pm 2.4\%$ of control NS to $132.50 \pm 2.7\%$ of BDNF 200 ng ($p < 0.001$) and $142.08 \pm 6.6\%$ of BDNF 300 ng ($p < 0.001$) at 120 min after drug administration, respectively (Fig. 2D). Furthermore, we tested effects of TrkB-Fc on BDNF-induced activities of dorsal horn WDR neurons. The results revealed that the BDNF-induced increase in the C-fiber responses of WDR neurons could be almost completely blocked by spinal application of TrkB-Fc (0.5 $\mu\text{g}/\mu\text{l}$) 30 min before BDNF administration ($105.55 \pm 3.1\%$ vs. $149.11 \pm 9.0\%$, TrkB-Fc vs. IgG at 120 min post-BDNF, Fig. 2E). Three representative examples illustrating effects of BDNF (300 ng) on the C-fiber responses of dorsal horn WDR neurons and effects of TrkB-Fc (or control IgG) on BDNF-induced activities of WDR neurons are displayed in Figs. 2A to C, respectively. Together with aforementioned behavioral data, these results suggest that spinal BDNF contributes to the development of long-lasting hyperexcitability of dorsal horn WDR neurons (i.e. central sensitization) as well as pain hypersensitivity following peripheral nerve injury.

BDNF induces LTP of C-fiber-evoked field potentials in the spinal dorsal horn via activation of GluN2B-NMDA receptors

In previous studies, we have found that GluN2B-NMDA receptor-dependent LTP in the spinal dorsal horn plays a crucial role in the development of long-lasting spinal neurons hyperexcitability and neuropathic pain (Qu et al., 2009; Xing et al., 2007), and increased BDNF in the spinal cord contributes to the pathogenesis of neuropathic pain by activation of GluN2B-NMDA receptors in rats suffered from spinal nerve injury (Geng et al., 2010). In this study, we further investigated whether spinal BDNF contributes to the induction of LTP at C-fiber synapses via activation of GluN2B-NMDA receptors in the spinal dorsal horn. As shown in Fig. 3A, spinal application of BDNF (300 ng) induced significant LTP of C-fiber-evoked field potentials with a long latency. The amplitude of C-fiber responses were significantly elevated above baseline approximately at 65 min ($123.9 \pm 4.1\%$, $p < 0.001$ vs. baseline), climbed gradually to a stable plateau ($160.8 \pm 2.5\%$, $p < 0.001$ vs. baseline) at 3–4 h after BDNF application, and persisted without decrement until our experiment termination. In contrast, no significant alteration was observed on C-fiber-evoked field potentials after spinal application of vehicle NS ($p > 0.05$, vs. baseline). When compared between the two

groups, mean C-fiber-evoked field potentials during 3–4 h following drug application was more larger in the BDNF group than that ($100.2 \pm 3.2\%$) in the NS group ($P < 0.001$, vs. NS group, Fig. 3E).

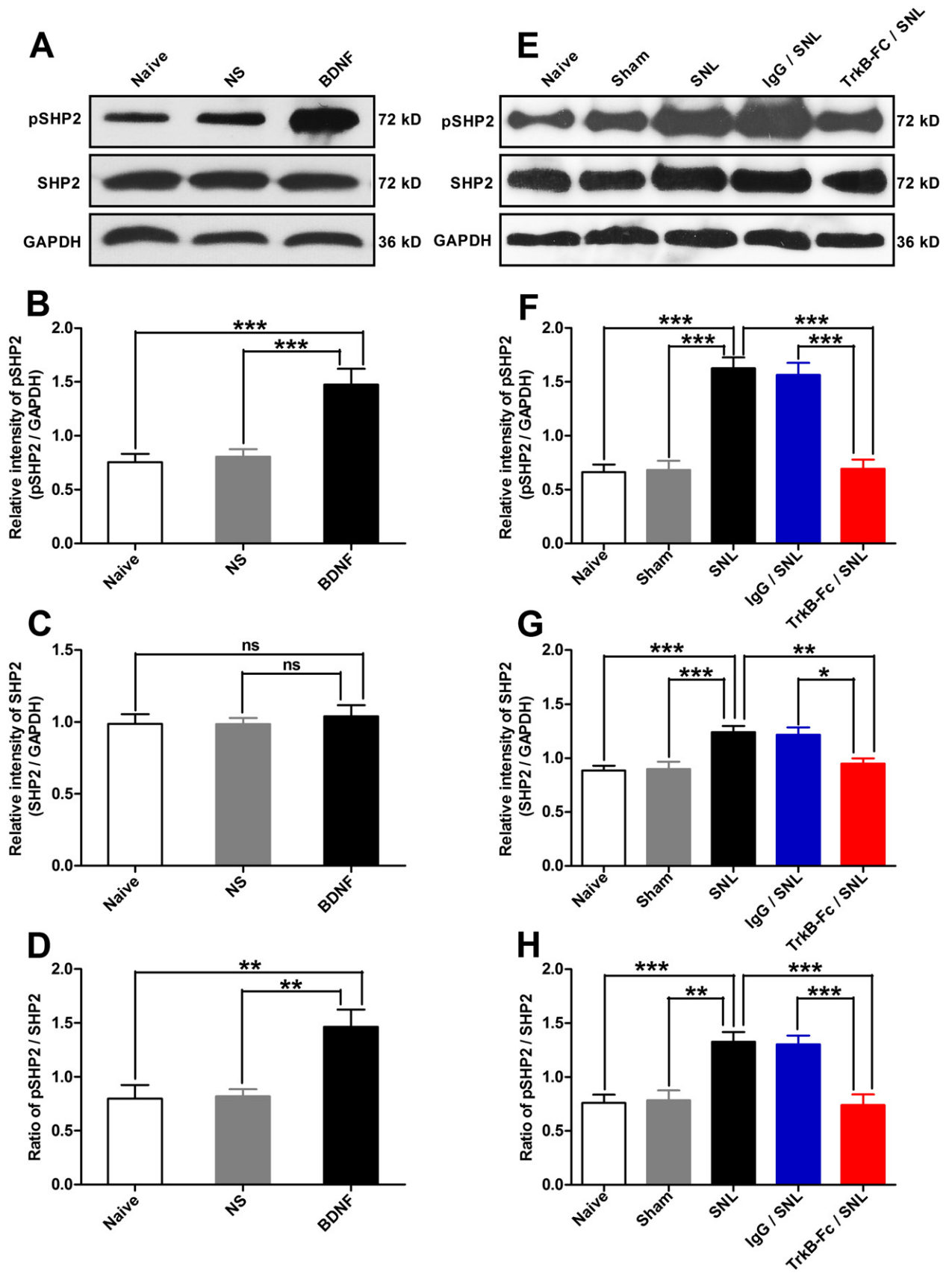
Next, we examined the role of TrkB receptor in BDNF-induced LTP using either TrkB-Fc, or genistein, a tyrosine kinase inhibitor. We found that perfusion of either TrkB-Fc (0.5 $\mu\text{g}/\mu\text{l}$) or genistein (5 $\mu\text{g}/\mu\text{l}$) for 30 min before application of BDNF could almost completely block the BDNF-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn, whereas perfusion of IgG or genistin (an inactive analogue of genistein) had no significant influence on the BDNF-induced LTP (Figs. 3B and C). When compared to the baseline responses (averaged at 30–0 min prior to drug), the mean C-fiber-evoked field potentials 3–4 h after BDNF application were $157.8 \pm 3.1\%$ in IgG treated rats and $101.9 \pm 3.2\%$ in TrkB-Fc treated rats ($P < 0.001$, vs. IgG group, Fig. 3F), and were $165.4 \pm 1.7\%$ in genistin treated rats and $103.7 \pm 4.3\%$ in genistein treated rats ($P < 0.001$, vs. genistin group, Fig. 3G).

In addition, we explored the role of GluN2B-NMDA receptor in BDNF-induced LTP using ifenprodil, a selective GluN2B-NMDA receptor inhibitor. Similarly, perfusion of ifenprodil (1 $\mu\text{g}/\mu\text{l}$) for 30 min before application of BDNF could also remarkably block the BDNF-induced LTP, while perfusion of vehicle DMSO had no significant effect (Fig. 3D). When compared to the baseline responses, the mean C-fiber-evoked field potentials 3–4 h after BDNF application were $161.7 \pm 2.1\%$ in DMSO treated rats and $106.1 \pm 2.9\%$ in ifenprodil treated rats ($P < 0.001$, vs. DMSO group, Fig. 3H). Taken together, these data suggest that BDNF/TrkB signaling in the spinal dorsal horn contributes to the induction of LTP at C-fiber synapses via activation of GluN2B-NMDA receptors.

BDNF-mediated LTP-like state by GluN2B-NMDA receptors activation contributes to the occlusion of spinal LTP elicited by HFS in SNL rats

It is suggested that peripheral nerve injury itself is likely to induce an LTP-like state, that is, preexisting sensitized conditions in the spinal dorsal horn, and occludes the subsequent development of spinal LTP elicited by electrical stimulation of the peripheral sensory afferents (Ohnami et al., 2011; Rygh et al., 2000). Previously, we have reported that spinal nerve injury induces a significant increase of BDNF in the spinal dorsal horn, and elevated BDNF contributes to the development of neuropathic pain by activation of GluN2B-NMDA receptors (Geng et al., 2010). Given that BDNF also induces spinal LTP through GluN2B-NMDA receptors activation (see Fig. 3), we hypothesized that increased BDNF in the spinal dorsal horn would contribute to the induction of LTP-like state (BDNF-LTP) in SNL rats via activation of GluN2B-NMDA receptors, and this kind of BDNF-LTP would occlude the subsequent development of spinal LTP elicited by electrical stimulation of the peripheral sensory afferents. As our expectation, we found that the high-frequency electrical stimulation (HFS)-induced LTP in the spinal dorsal horn was markedly occluded in SNL rats compared to sham-operated rats (Fig. 4A). When compared to the baseline responses (averaged at 30–0 min prior to HFS), mean C-fiber-evoked field potentials 5–240 min after HFS were $193.5 \pm 6.2\%$ in sham-operated rats and $135.3 \pm 4.0\%$ in SNL rats ($P < 0.001$, vs. sham group, Fig. 4A, bar graph). However, in those SNL rats either pretreated with TrkB-Fc or ifenprodil (see Fig. 4E), which is sufficient to prevent BDNF-induced spinal LTP in intact rats (see Fig. 3) and alleviate neuropathic pain syndrome in SNL rats (see (Qu et al.,

Fig. 6. Intrathecal injection of BDNF enhances the expression of dorsal horn phosphorylated SHP2, while pre-treatment with TrkB-Fc prevents the nerve injury-induced increase of phosphorylated SHP2 in SNL rats. (A–D) Western blot of phosphorylated SHP2 (pSHP2) and SHP2 expression in synaptosomal fraction extracted from the spinal dorsal horn tissue in naïve, NS-treated, and BDNF-treated rats. (A) Representative of Western blot bands for pSHP2 (upper), SHP2 (middle) and GAPDH (lower). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as internal control. (B–D) analysis of the relative intensity of pSHP2 (B), SHP2 (C), and ratio of pSHP2/SHP2 (D). Note the expression of pSHP2 but not SHP2 is statistically increased, therefore the ratio of pSHP2/SHP2 is markedly elevated in BDNF-treated rats as compared to naïve and NS-treated rats, respectively. $**P < 0.01$, $***P < 0.001$, one-way ANOVA, $n = 5/\text{group}$. (E–H) Western blot of pSHP2 and SHP2 expression in synaptosomal fraction extracted from the spinal dorsal horn tissue in naïve, SNL, sham-operated rats, and in SNL rats pre-treated with TrkB-Fc or IgG. (E) Representative of Western blot bands for pSHP2 (upper), SHP2 (middle) and GAPDH (lower). (F–H) analysis of the relative intensity of pSHP2 (F), SHP2 (G), and ratio of pSHP2/SHP2 (H). Note the expression of pSHP2, SHP2 as well as ratio of pSHP2/SHP2 are significantly increased in SNL rats as compared to naïve and sham-operated rats, respectively. Moreover, all the SNL-induced increase of pSHP2, SHP2 and ratio of pSHP2/SHP2 are almost completely rescued by preemptive application of TrkB-Fc. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, one-way ANOVA, $n = 6/\text{group}$.



2009) and Figs. 1G and I), the occluded spinal LTP elicited by HFS in SNL rats was significantly rescued (Figs. 4B and C). When compared to the baseline responses (averaged at 30–0 min prior to HFS), the mean C-fiber-evoked field potentials 5–240 min after HFS were $134.3 \pm 7.7\%$ in IgG pretreated rats and $195.5 \pm 8.6\%$ in TrkB-FC pretreated rats ($P < 0.001$, vs. IgG group, Fig. 4B, bar graph), and were $137.9 \pm 5.2\%$ in DMSO pretreated rats and $200.8 \pm 7.9\%$ in ifenprodil pretreated rats ($P < 0.001$, vs. DMSO group, Fig. 4C, bar graph). A summary of the mean C-fiber-evoked field potentials 5–240 min after HFS in all 6 groups above is shown in Fig. 4D. Together these data with aforementioned findings, we suggest that BDNF-mediated LTP-like state via activation of GluN2B-NMDA receptors likely contributes to the occlusion of spinal LTP elicited by HFS in SNL rats.

BDNF contributes to the functional up-regulation of GluN2B-NMDA receptors in the spinal dorsal horn of SNL rats

To further provide direct evidence for our hypothesis that increased BDNF in the spinal dorsal horn after SNL surgery activates GluN2B-NMDA receptors, which subsequently induces an LTP-like state, and occludes the HFS-induced spinal LTP, we first explored whether BDNF would induce functional up-regulation of GluN2B-NMDA receptors by detecting the receptors expression in synaptosomal fraction extracted from the spinal dorsal horn tissue, which is usually performed to assess functional expression of receptors at a synapse. BDNF was intrathecally delivered to intact rats as described in aforementioned behavioral experiments (see Fig. 1D), and Western blot analysis was carried out 7 days after drug injection and behavioral test. As shown in Fig. 5A, spinal application of BDNF induced a marked up-regulation of NMDA receptor GluN2B in synaptosomal fraction of the spinal dorsal horn. The relative optical band density of GluN2B immunoreactivity was prominently increased ($148.8 \pm 15.1\%$ of naïve and $161.1 \pm 13.1\%$ of NS, $p < 0.001$) in BDNF-treated rats compared to naïve and NS-treated rats.

Next, we examined whether SNL surgery itself would induce a functional up-regulation of GluN2B-NMDA receptors in the spinal dorsal horn, and if so, whether this enhancement of GluN2B was resulted from an exertion of increased BDNF emerged after spinal nerve injury. As our expectation, we observed an obvious functional up-regulation of GluN2B-NMDA receptors in the spinal dorsal horn 7 days after SNL surgery. The relative optical band density of GluN2B immunoreactivity in synaptosomal fraction of the spinal dorsal horn was dramatically increased ($186.7 \pm 19.1\%$ of naïve and $181.1 \pm 17.4\%$ of sham, $p < 0.001$, Fig. 5B) in SNL rats compared to naïve and sham-operated rats. However, in those SNL rats pre-treated with TrkB-FC (see Fig. 1E), the SNL-induced functional up-regulation of GluN2B-NMDA receptors in the spinal dorsal horn was almost completely restored. The relative optical band density of GluN2B immunoreactivity in synaptosomal fraction of the spinal dorsal horn was significantly decreased in TrkB-FC pretreated rats ($58.5 \pm 7.4\%$ of SNL) compared to control IgG pretreated rats ($100.7 \pm 11.7\%$ of SNL, $p < 0.001$, Fig. 5B). Taken together, these results suggest that increased BDNF in SNL rats induces functional up-regulation of GluN2B-NMDA receptors in the spinal dorsal horn following spinal nerve injury.

BDNF contributes to the phosphorylation of SHP2 in the spinal dorsal horn of SNL rats following spinal nerve injury

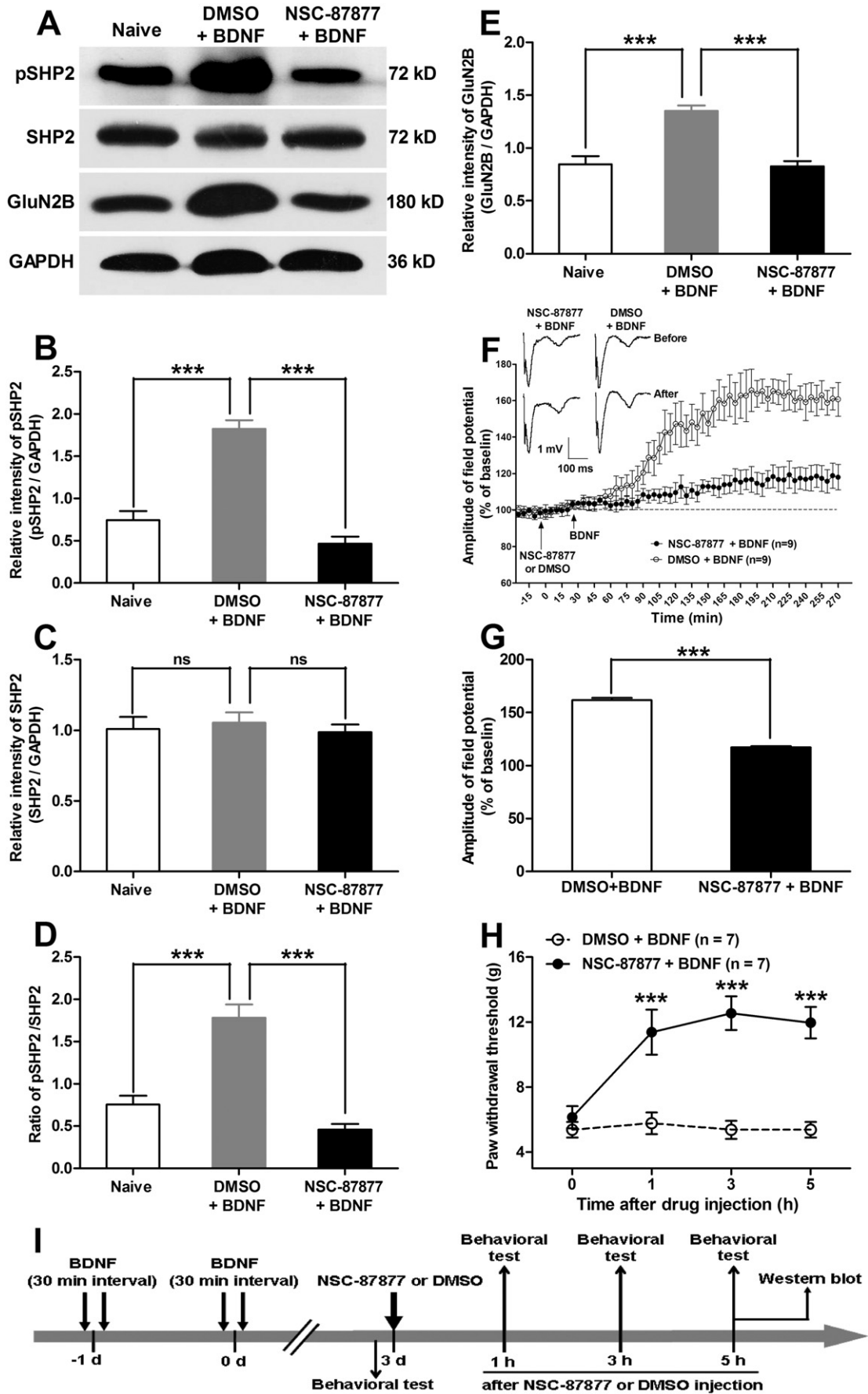
To clarify the underlying mechanisms by which how BDNF activates GluN2B-NMDA receptors in the spinal dorsal horn following spinal nerve injury, we focused on SHP2, a Src homology-2 (H2) domain-containing protein tyrosine phosphatase-2, which has TrkB/TrkB and GluN2B-NMDA signaling pathway (Araki et al., 2000; Easton et al., 2006; Lin et al., 1999; Neel et al., 2003; Okada et al., 1996; Peng et al., 2012). We discovered that in intact rats, 7 days after intrathecal administration of BDNF (100 ng, twice per day for 2 days, see Fig. 1D), expression of phosphorylated SHP2 (pSHP2) ($223.8 \pm 51.0\%$ of naïve and $199.6 \pm 37.3\%$ of NS, $p < 0.001$) but not SHP2 ($112.2 \pm 17.1\%$ of naïve and $107.5 \pm 10.6\%$ of NS, $p > 0.05$) in synaptosomal fraction of the spinal dorsal horn was significantly increased, and thus ratio of pSHP2/SHP2 was markedly elevated ($217.8 \pm 41.3\%$ of naïve and $194.6 \pm 37.9\%$ of NS, $p < 0.001$) (Figs. 6A–D).

While in SNL rats, we observed a remarkable increase both in expression of pSHP2 ($299.7 \pm 44.2\%$ of naïve and $328.2 \pm 76.8\%$ of sham, $p < 0.001$) and SHP2 ($203.6 \pm 28.7\%$ of naïve and $229.9 \pm 58.0\%$ of sham, $p < 0.001$) in synaptosomal fraction of the spinal dorsal horn 7 days after SNL surgery, and also, a prominent increase was observed on ratio of pSHP2/SHP2 ($150.6 \pm 11.4\%$ of naïve and $153.9 \pm 17.0\%$ of sham, $p < 0.001$ vs. naïve, $p < 0.01$ vs. sham) (Figs. 6E to H). In addition, we found that the SNL-induced increase in both pSHP2 and SHP2 could be significantly restored by pre-treatment with TrkB-FC (see Fig. 1E) to those rats suffered from SNL surgery. The relative optical band density of pSHP2 immunoreactivity in synaptosomal fraction of the spinal dorsal horn was significantly decreased in TrkB-FC pre-treated rats ($45.5 \pm 7.2\%$ of SNL) compared to control IgG pretreated rats ($102.3 \pm 9.8\%$ of SNL, $p < 0.001$). Similarly, the immunoreactivity of SHP2 was more lower in TrkB-FC pre-treated rats ($79.2 \pm 6.2\%$ of SNL) than that in IgG pre-treated rats ($100.5 \pm 7.6\%$ of SNL, $p < 0.05$), and the ratio of pSHP2/SHP2 was reduced in TrkB-FC pretreated rats ($61.7 \pm 11.2\%$ of SNL) compared to IgG pre-treated rats ($102.5 \pm 8.4\%$ of SNL, $p < 0.001$) (Figs. 6E–H). These results indicate that increased BDNF in the spinal dorsal horn may activate SHP2 in rats following peripheral nerve injury.

BDNF-induced SHP2 phosphorylation in the spinal dorsal horn is required for subsequent GluN2B-NMDA receptors up-regulation and spinal LTP induction, and also for pain allodynia elicitation.

To determine whether spinal SHP2 is involved in BDNF-mediated activation of GluN2B-NMDA receptors as well as in the induction of spinal LTP and post-injury pain hypersensitivity, we first examined effects of NSC-87877, a potent SHP2 protein tyrosine phosphatase inhibitor (Chen et al., 2006; Peng et al., 2012) on BDNF-induced GluN2B-NMDA receptors activation as well as spinal LTP induction and pain allodynia elicitation in intact rats. As shown in Fig. 7, intrathecal administration of NSC-87877 (see Fig. 7I) could dramatically inhibit the BDNF-induced SHP2 phosphorylation, and ultimately inhibit the BDNF-induced GluN2B up-regulation and spinal LTP induction, and also attenuate the BDNF-evoked pain allodynia. The BDNF-induced expression of pSHP2

Fig. 7. Spinal application of NSC-87877 inhibits BDNF-evoked SHP2 phosphorylation as well as GluN2B up-regulation and spinal LTP induction, and pain allodynia elicitation in intact rats. (A–E) Western blot of pSHP2, SHP2 and GluN2B expression in synaptosomal fraction extracted from the spinal dorsal horn tissue in naïve rats, and in rats received intrathecal injection of NSC-87877 or DMSO on day 3 after BDNF administration. (A) Representative of Western blot bands for pSHP2, SHP2 and GluN2B. GAPDH is used as internal control. (B–E) analysis of the relative intensity of pSHP2 (B), SHP2 (C), ratio of pSHP2/SHP2 (D) and GluN2B (E). Note that intrathecal administration of NSC-87877 dramatically inhibits the BDNF-induced increase of pSHP2 (B), ratio of pSHP2/SHP2 (D) and up-regulation of GluN2B expression (E). *** $P < 0.001$, one-way ANOVA, $n = 7$ /group. (F and G) Effects of NSC-87877 on BDNF-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn. Note that perfusion of NSC-87877 (2.5 ng/ μ l) for 30 min before spinal application of BDNF (300 ng) remarkably inhibits the BDNF-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn (F). Traces at top are recorded at 10 min before and 3 h after BDNF application, respectively. (G) Comparison of mean C-fiber-evoked field potentials during 3–4 h after BDNF application between the NSC-87877 and the DMSO group. *** $P < 0.001$, two-tailed unpaired t-test, $n = 9$ /group. (H) Effects of NSC-87877 on BDNF-evoked pain allodynia. Note the decreased paw withdrawal threshold induced by spinal administration of BDNF is significantly restored in NSC-87877-treated rats at 1, 3 and 5 h after NSC-87877 application, suggesting that intrathecal injection of NSC-87877 can dramatically attenuate the BDNF-evoked pain allodynia. *** $P < 0.001$, two-way ANOVA, $n = 7$ /group. (I) Scheme of the experimental procedure. BDNF (100 ng) was intrathecally administrated to rats twice per day at a 30 min-interval, repeated for 2 days, and then NSC-87877 (2.5 ng/ μ l) was intrathecally applied on day 3 after the last BDNF injection. The PWT of rat was measured before and at 1, 3 and 5 h after NSC-87877 application, respectively. Western blot was performed after behavioral test.



(27.3 ± 5.9% of DMSO-treated rats, $p < 0.001$) but not SHP2 (95.6 ± 7.4% of DMSO-treated rats, $p > 0.05$) in synaptosomal fraction of the spinal dorsal horn was prominently decreased in NSC-87877-treated rats as compared to DMSO-treated rats, and also the ratio of pSHP2/SHP2 was decreased obviously in NSC-87877-treated rats (28.3 ± 5.8% of DMSO-treated rats, $p < 0.001$) (Figs. 7A to D). Accordingly, the BDNF-induced up-regulation of GluN2B in synaptosomal fraction of the spinal dorsal horn was remarkably inhibited in NSC-87877-treated rats (62.0 ± 3.9% of DMSO-treated rats, $p < 0.001$, Fig. 7E) as compared to DMSO-treated rats. Similarly, the BDNF-induced LTP was also prevented by spinal application of NSC-87877. When compared to the baseline responses (averaged at 30–0 min prior to drug), the mean C-fiber-evoked field potentials 3–4 h after BDNF application were 161.7 ± 2.1% in DMSO-treated rats and 117.5 ± 1.1% in NSC-87877-treated rats ($P < 0.001$, vs. DMSO group, Figs. 7F and G). In addition, the BDNF-evoked pain allodynia was also attenuated by intrathecal administration of NSC-87877. The decreased paw withdrawal threshold (in gram) induced by BDNF application was significantly restored in NSC-87877-treated rats at 1 h (11.38 ± 1.4 NSC-87877 vs. 5.78 ± 0.7 DMSO, $P < 0.001$), 3 h (12.55 ± 1.0 NSC-87877 vs. 5.38 ± 0.6 DMSO, $P < 0.001$) and 5 h (11.96 ± 0.9 NSC-87877 vs. 5.37 ± 0.5 DMSO, $P < 0.001$) after NSC-87877 administration (Fig. 7H). These data indicate that the BDNF-induced SHP2 phosphorylation in the spinal dorsal horn is required for subsequent GluN2B-NMDA receptors activation and spinal LTP induction, and ultimately for pain allodynia elicitation.

Inhibition of SHP2 phosphorylation in the spinal dorsal horn reduces the nerve injury-evoked GluN2B-NMDA receptors up-regulation and spinal LTP occlusion, and also alleviates pain allodynia in neuropathic rats

Next, we examined effects of NSC-87877 on nerve injury-induced GluN2B-NMDA receptors up-regulation as well as spinal LTP induction and pain allodynia elicitation in SNL rats. As shown in Fig. 8, intrathecal administration of NSC-87877 (see Fig. 8I) could dramatically inhibit SNL-induced SHP2 phosphorylation, and subsequently inhibit SNL-induced GluN2B up-regulation and spinal LTP occlusion, and also alleviate SNL-evoked pain allodynia. The SNL-induced expression of both pSHP2 (24.5 ± 2.1% of DMSO-treated rats, $p < 0.001$) and SHP2 (53.7 ± 3.7% of DMSO-treated rats, $p < 0.001$) in synaptosomal fraction of the spinal dorsal horn was prominently decreased in NSC-87877-treated rats compared to DMSO-treated rats, and ultimately the ratio of pSHP2/SHP2 was decreased obviously (46.4 ± 4.7% of DMSO-treated rats, $p < 0.001$) (Figs. 8A to D). Accordingly, the SNL-induced up-regulation of GluN2B in synaptosomal fraction of the spinal dorsal horn was remarkably inhibited in NSC-87877-treated rats (51.9 ± 3.0% of DMSO-treated rats, $p < 0.001$, Fig. 8E) compared to DMSO-treated rats. Similarly, the occlusion of HFS-induced spinal LTP by SNL surgery was also rescued by spinal application of NSC-87877. When compared to the baseline responses (averaged at 30–0 min prior to drug), the mean C-fiber-evoked field potentials 3–4 h after SNL surgery were 133.6 ± 4.3% in DMSO-treated rats and 209.7 ± 3.8% in NSC-87877-treated rats ($P < 0.001$, vs. DMSO group, Figs. 8F and G). In addition, the SNL-evoked pain allodynia was also attenuated by intrathecal administration of NSC-87877. The decreased paw withdrawal threshold (in gram) induced by SNL surgery was significantly restored in NSC-

87877-treated rats at 1 h (7.85 ± 1.2 NSC-87877 vs. 4.30 ± 0.5 DMSO, $P < 0.01$), 3 h (11.09 ± 1.4 NSC-87877 vs. 3.71 ± 0.4 DMSO, $P < 0.001$) and 5 h (7.57 ± 0.6 NSC-87877 vs. 3.97 ± 0.3 DMSO, $P < 0.01$) after NSC-87877 application (Fig. 8H). These data indicate that inhibition of SHP2 phosphorylation in the spinal dorsal horn reduces the SNL-evoked GluN2B-NMDA receptors up-regulation and spinal LTP occlusion, and ultimately alleviates pain allodynia in neuropathic rats.

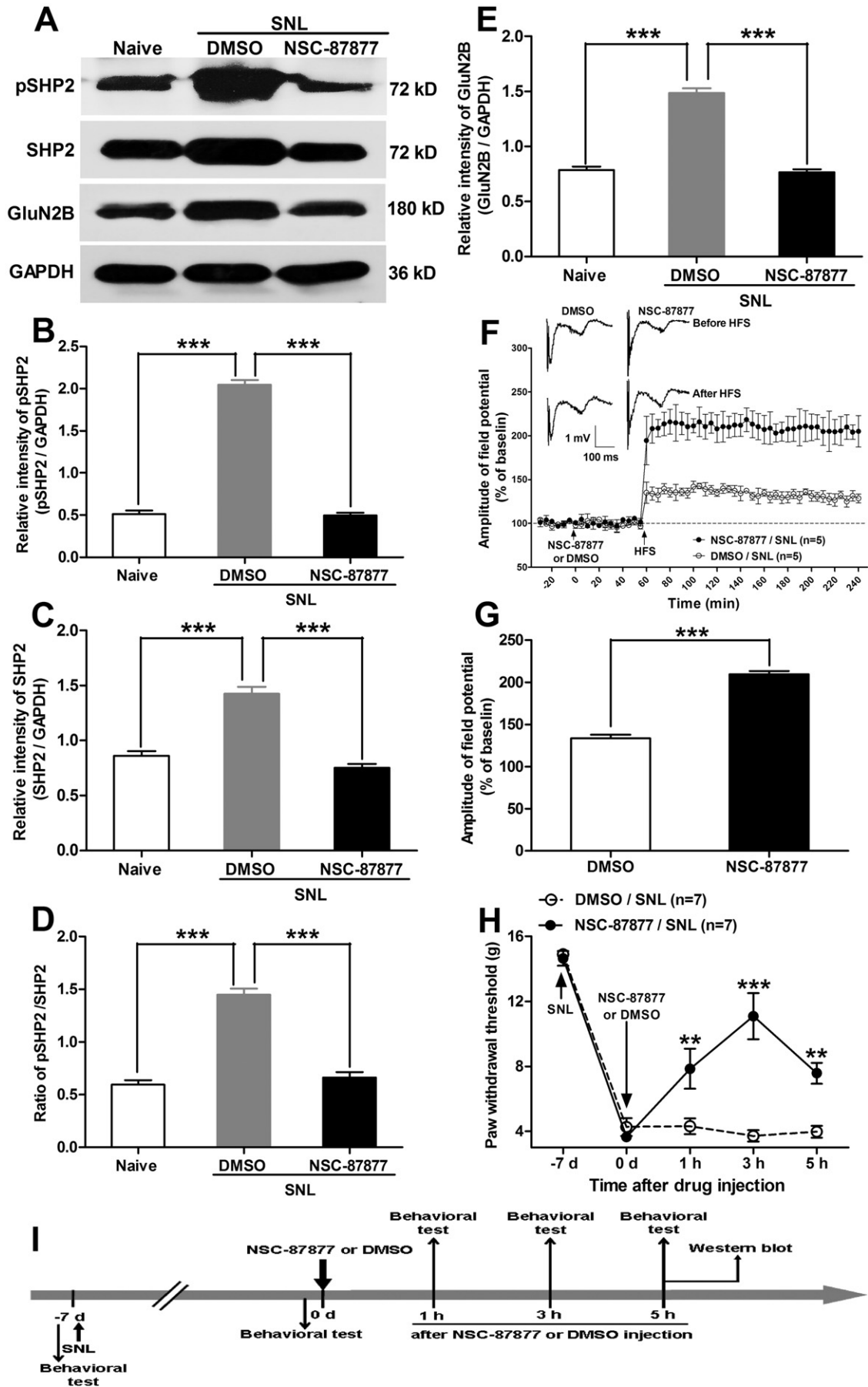
Knockdown of SHP2 prevents BDNF-induced GluN2B-NMDA receptors up-regulation and spinal LTP induction, and also attenuates BDNF-induced pain allodynia

Furthermore, we examined effects of SHP2 knockdown on BDNF-mediated NMDA receptor GluN2B up-regulation as well as spinal LTP induction and pain allodynia elicitation in intact rats. As shown in Fig. 9, knockdown of SHP2 could remarkably inhibit the BDNF-induced NMDA receptor GluN2B up-regulation and spinal LTP induction, and also attenuate the BDNF-induced pain allodynia. We found that intrathecal injection of LV-shSHP2 (see Fig. 9I) indeed inhibited the increase of SHP2 (0.55 ± 0.09 LV-shSHP2 vs. 1.21 ± 0.11 LV-GFP and 0.77 ± 0.06 naïve, $p < 0.001$) but not SHP1 (0.97 ± 0.05 LV-shSHP2 vs. 1.06 ± 0.04 LV-GFP and 1.01 ± 0.06 naïve, $p > 0.05$) in synaptosomal fraction of the spinal dorsal horn after spinal application of BDNF (Figs. 9A to C). Accordingly, the expression of GluN2B protein in synaptosomal fraction of the spinal dorsal horn was also decreased dramatically in LV-shSHP2-treated rats (0.81 ± 0.06) compared to LV-GFP-treated rats (1.31 ± 0.05) after spinal administration of BDNF ($p < 0.001$, Figs. 9A and D). Similarly, the BDNF-induced LTP was also prevented by spinal application of LV-shSHP2. When compared to the baseline responses (averaged at 30–0 min prior to drug), the mean C-fiber-evoked field potentials 3–4 h after BDNF application were 167.2 ± 1.4% in LV-GFP-treated rats and 116.2 ± 1.8% in LV-shSHP2-treated rats ($P < 0.001$, vs. LV-GFP group, Figs. 9E and F). As expected, the BDNF-evoked pain allodynia was also attenuated by spinal application of LV-shSHP2. The decreased PWT (in gram) induced by BDNF was significantly restored in LV-shSHP2-treated rats from day 1 (13.51 ± 0.8 LV-shSHP2 vs. 5.69 ± 0.7 LV-GFP, $P < 0.001$) to day 7 (11.91 ± 1.3 LV-shSHP2 vs. 6.84 ± 1.0 LV-GFP, $P < 0.001$) after BDNF application (Fig. 9G). In addition, no significant influence was observed on the locomotor function of rats after spinal application of LV-shSHP2 compared to LV-GFP ($p > 0.05$, Fig. 9H).

Knockdown of SHP2 prevents SNL-induced GluN2B-NMDA receptors up-regulation and spinal LTP occlusion, and also attenuates pain allodynia in neuropathic rats

Finally, we examined effects of SHP2 knockdown on nerve injury-induced GluN2B-NMDA receptors activation as well as spinal LTP induction and pain allodynia elicitation in SNL rats. As shown in Fig. 10, intrathecal injection of LV-shSHP2 (see Fig. 10G) could significantly inhibit the increase of SHP2 (0.78 ± 0.04 LV-shSHP2 vs. 1.46 ± 0.04 LV-GFP and 0.80 ± 0.06 naïve, $p < 0.001$) but not SHP1 (0.98 ± 0.04 LV-shSHP2 vs. 1.01 ± 0.04 LV-GFP and 1.01 ± 0.03 naïve, $p > 0.05$) in synaptosomal fraction of the spinal dorsal horn after SNL surgery (Figs. 10A to C). Moreover, the expression of GluN2B

Fig. 8. Spinal application of NSC-87877 inhibits nerve injury-evoked SHP2 phosphorylation as well as GluN2B up-regulation and spinal LTP occlusion, and pain allodynia development in SNL rats. (A–E) Western blot of pSHP2, SHP2 and GluN2B expression in synaptosomal fraction extracted from the spinal dorsal horn tissue in naïve rats, and in SNL rats received NSC-87877 or DMSO treatment. (A) Representative of Western blot bands for pSHP2, SHP2 and GluN2B. GAPDH is used as internal control. (B–E) analysis of the relative intensity of pSHP2 (B), SHP2 (C), ratio of pSHP2/SHP2 (D) and GluN2B (E). Note that intrathecal administration of NSC-87877 dramatically inhibits SNL-induced increase of pSHP2 (B), SHP2 (C), ratio of pSHP2/SHP2 (D), and up-regulation of GluN2B expression (E). *** $P < 0.001$, one-way ANOVA, $n = 5$ /group. (F and G) Effects of NSC-87877 on HFS-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn. Note that perfusion of NSC-87877 (2.5 ng/μl) for 30 min before HFS of the sciatic nerve remarkably inhibits the decrease of HFS-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn in SNL rats (F). Traces at top are recorded at 10 min before and 3 h after HFS of the sciatic nerve, respectively. (G) Comparison of mean C-fiber-evoked field potentials during 3–4 h after HFS of the sciatic nerve between the NSC-87877 and the DMSO group. **** $P < 0.001$, two-tailed unpaired t-test, $n = 5$ /group. (H) Effects of NSC-87877 on SNL-evoked pain allodynia. Note the decreased paw withdrawal threshold induced by SNL surgery is significantly rescued in NSC-87877-treated rats at 1, 3 and 5 h after NSC-87877 application, suggesting that intrathecal injection of NSC-87877 attenuates SNL-evoked pain allodynia. ** $P < 0.01$, *** $P < 0.001$, two-way ANOVA, $n = 7$ /group. (I) Scheme of the experimental procedure. On day 7 after SNL operation, NSC-87877 (2.5 ng/μl) was intrathecally delivered to rats exhibiting mechanical allodynia. The PWT was measured before SNL surgery and NSC-87877 injection, and then at 1, 3 and 5 h after NSC-87877 application, respectively. Western blot was performed after behavioral test.



protein in synaptosomal fraction of the spinal dorsal horn was decreased markedly in LV-shSHP2-treated rats (0.70 ± 0.05) compared to LV-GFP-treated rats (1.53 ± 0.07) after SNL surgery ($p < 0.001$, Figs. 10A and D). And also, the occlusion of HFS-induced spinal LTP by SNL surgery was obviously prevented by spinal application of LV-shSHP2. When compared to the baseline responses (averaged at 30–0 min prior to drug), the mean C-fiber-evoked field potential 3–4 h after HFS was $131.4 \pm 2.9\%$ in LV-GFP-treated rats and $187.9 \pm 2.2\%$ in LV-shSHP2-treated rats ($P < 0.001$, vs. LV-GFP group, Fig. 10E). Similarly, intrathecal delivery of LV-shSHP2 prior SNL surgery could also prominently attenuate the nerve injury-induced pain allodynia in neuropathic rats. The decreased PWT (in gram) induced by nerve injury was significantly prevented in LV-shSHP2-treated rats from day 1 (13.39 ± 1.2 LV-shSHP2 vs. 7.47 ± 1.5 LV-GFP, $P < 0.001$) to day 7 (11.93 ± 1.3 LV-shSHP2 vs. 4.22 ± 0.9 LV-GFP, $P < 0.001$) after SNL surgery (Fig. 10F). These data suggest that knockdown of SHP2 in the spinal dorsal horn prevents the nerve injury-evoked GluN2B-NMDA receptors up-regulation and spinal LTP occlusion, and also attenuates the pain allodynia in neuropathic rats.

Discussion

In this study, we present valid evidence showing that the BDNF-induced SHP2 phosphorylation in the spinal dorsal horn is required for subsequent GluN2B-NMDA receptors up-regulation and spinal LTP induction, and ultimately for neuropathic pain development following spinal nerve ligation.

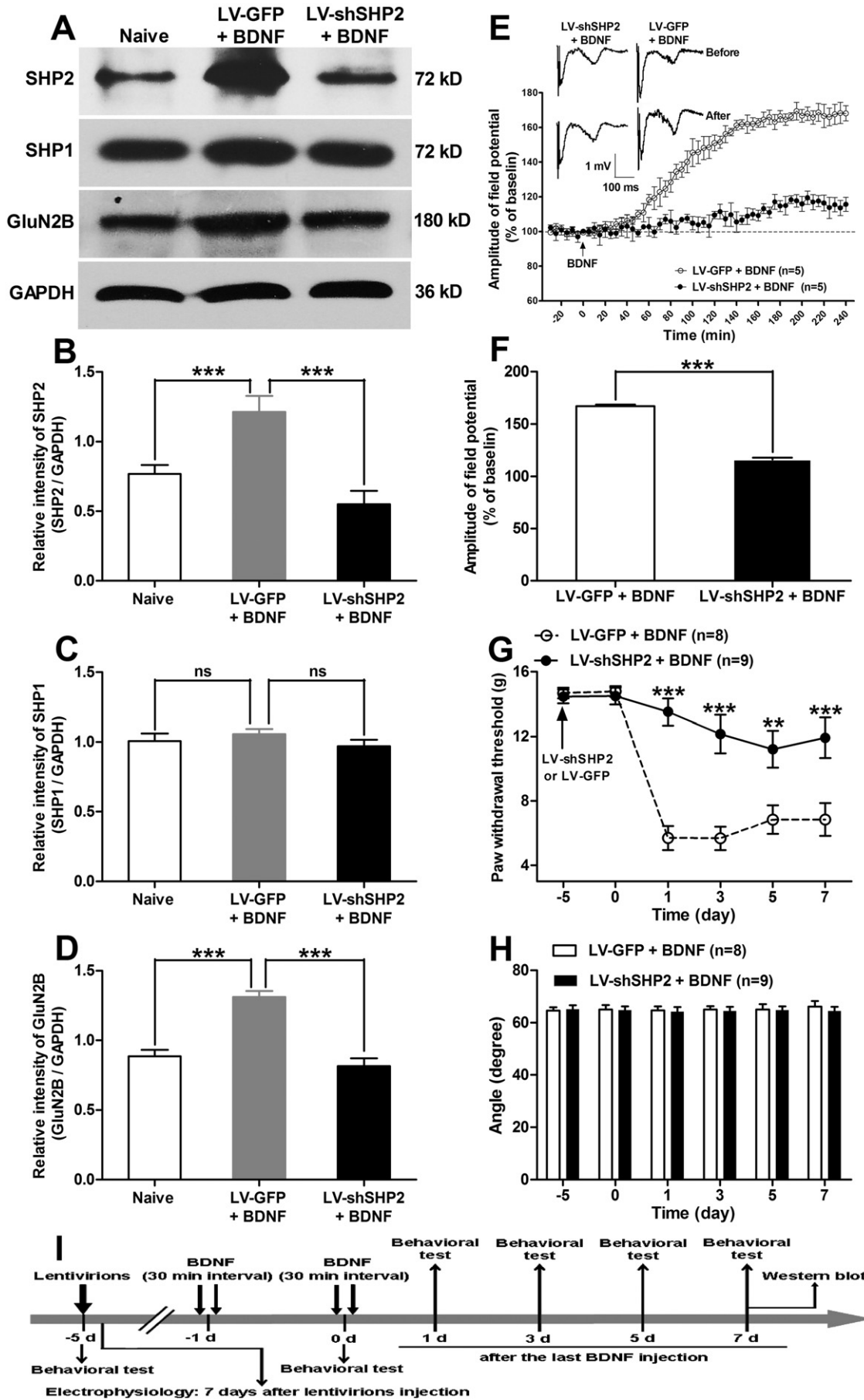
Contribution of BDNF to the development of neuropathic pain via induction of NMDA receptor GluN2B-dependent LTP in the spinal dorsal horn

Previously, we and others have reported that the concentration of BDNF in the lumbar spinal dorsal horn increases in rats with thermal hyperalgesia after loose ligation of the sciatic nerve (Miletic and Miletic, 2002), and the expression of BDNF protein enhances in the superficial dorsal horn of the spinal cord following nerve injury (Geng et al., 2010; Ha et al., 2001; Zhou et al., 1999). In the present study, we first investigated whether increased BDNF in the spinal dorsal horn plays a crucial role in the development of neuropathic pain in rats suffered from nerve injury. Consistent with previous findings that the BDNF/TrkB signaling pathway is involved in synaptic mechanisms underlying both memory and pain (Bramham and Messaoudi, 2005; Leal et al., 2014; Malcangio and Lessmann, 2003; Merighi et al., 2008b; Smith, 2014; Wang et al., 2009), we here provide pharmacological evidence confirming the contribution of spinal BDNF to the pathogenesis of neuropathic pain by showing that intrathecal administration of exogenous BDNF mimics pain allodynia in intact rats, while spinal application of TrkB-Fc to scavenge endogenous BDNF, either pre-SNL or post-SNL surgery, attenuates pain hypersensitivity in neuropathic rats. To further determine whether increased BDNF in the spinal dorsal horn participates in the development of central sensitization, a potentiated synaptic efficacy and central neuron hyperexcitability that underlying pathogenesis of neuropathic pain following peripheral nerve injury

(Berger et al., 2011; Campbell and Meyer, 2006; Fornasari, 2012; Latremoliere and Woolf, 2009; Woolf, 2011), we also examined effects of spinal BDNF on the C-fiber responses of dorsal horn WDR neurons, which are highly related to nociceptive transmission (Qu et al., 2009; Rygh et al., 2000). We discovered that spinal application of BDNF induced long-lasting hyperexcitability of dorsal horn WDR neurons as assessed by measuring the C-fiber evoked discharges, and also, this kind hyperexcitability of dorsal horn WDR neurons induced by BDNF could be almost completely blocked by pre-treatment with TrkB-Fc. Together with these results with aforementioned behavioral data, we suggest that increased BDNF in the spinal dorsal horn contributes to the development of long-lasting hyperexcitability of dorsal horn WDR neurons (i.e. central sensitization) as well as pain hypersensitivity following spinal nerve injury.

It is well established that BDNF exerts its effects via interactions with other receptors or ion channels (Chao, 2003; Ren and Dubner, 2007; Rose et al., 2004; Schinder and Poo, 2000), and GluN2B-NMDA receptor is regarded as one of the targets of BDNF-induced modulation of synaptic plasticity (Caldeira et al., 2007b; Crozier et al., 1999; Kim et al., 2006; Levine and Kolb, 2000; Lin et al., 1998, 1999). For example, BDNF increases the phosphorylation of GluN1 and GluN2B subunits of NMDA receptors in isolated postsynaptic densities (PSDs) from cortical, hippocampal and the spinal cord (Di et al., 2001; Lin et al., 1998, 1999; Suen et al., 1997), and enhances NMDA receptors activity (Kim et al., 2006; Levine and Kolb, 2000). Phosphorylation of GluN2B by Fyn is suggested to contribute to the increase of glutamatergic synaptic transmission induced by BDNF (Alder et al., 2005; Xu et al., 2006), and tyrosine phosphorylation of GluN2B is also considered to underlie the induction of LTP in the hippocampus (Nakazawa et al., 2001, 2002) as well as the maintenance of neuropathic pain (Abe et al., 2005). In addition, the BDNF-mediated potentiation of excitatory transmission in the spinal dorsal horn appears to be associated with the activation of postsynaptic NMDA receptors (Garraway et al., 2003; Merighi et al., 2008a). The release of BDNF within the spinal cord results in phosphorylation and potentiation of NMDA receptors on the spinal cord neurons (Kerr et al., 1999; Obata and Noguchi, 2006; Slack et al., 2004; Slack and Thompson, 2002), and this effect represents a possible mechanism by which BDNF mediates LTP in the spinal dorsal horn (Zhou et al., 2008, 2011) as well as central sensitization in nociceptive pathways (Garraway et al., 2003; Malcangio and Lessmann, 2003; Ruscheweyh et al., 2011; Wang et al., 2009). In previous studies, we have found that increased BDNF in the spinal cord contributes to the pathogenesis of neuropathic pain by activation of GluN2B-NMDA receptors in rats suffered from spinal nerve injury (Geng et al., 2010), and spinal GluN2B-NMDA receptors play a crucial role in the development of central sensitization and neuropathic pain via induction of dorsal horn LTP (Qu et al., 2009; Xing et al., 2007). In this study, we provide several lines of evidence confirming our hypothesis that the induction of spinal LTP at C-fiber synapses via activation of GluN2B-NMDA receptors may be the underlying mechanism by which the increased BDNF in the spinal dorsal horn plays its roles in the development of central sensitization and pain hypersensitivity following nerve injury. Firstly, we provide electrophysiological evidence demonstrating that spinal BDNF contributes to the

Fig. 9. Knockdown of SHP2 in the spinal cord by intrathecal delivery of LV-shSHP2 prevents BDNF-evoked GluN2B up-regulation as well as spinal LTP induction and pain allodynia elicitation in intact rats. (A–D) Western blot of SHP2, SHP1, and GluN2B expression in synaptosomal fraction extracted from the spinal dorsal horn tissue in naïve rats, and in LV-shSHP2-infected or LV-GFP-infected rats received spinal BDNF. (A) Representative of Western blot bands for SHP2, SHP1, and GluN2B. GAPDH is used as internal control. (B–D) analysis of the relative intensity of SHP2 (B), SHP1 (C), and GluN2B (D). Note that the expression of SHP2 but not SHP1 is statistically decreased in LV-shSHP2 infected rats compared to LV-GFP infected rats. Similarly, intrathecal delivery of LV-shSHP2 significantly inhibits the expression of GluN2B after intrathecal injection of BDNF. $***P < 0.001$, one-way ANOVA, $n = 7$ /group. (E and F) Effects of LV-shSHP2 on BDNF-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn. Note that intrathecal delivery of LV-shSHP2 remarkably inhibits BDNF-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn (E). Traces at top are recorded at 10 min before and 3 h after BDNF application, respectively. (F) Comparison of mean C-fiber-evoked field potentials during 3–4 h after BDNF application between the LV-shSHP2 and the LV-GFP group. $***P < 0.001$, two-tailed unpaired t-test, $n = 5$ /group. (G and H) Effects of LV-shSHP2 on BDNF-evoked pain allodynia (G) and locomotor function (H). Note the decreased paw withdrawal threshold induced by spinal administration of BDNF is significantly restored in LV-shSHP2-infected rats compared to LV-GFP-infected rats, suggesting that knockdown of SHP2 by SHP2 siRNA prevents BDNF-evoked pain allodynia in intact rats. $**P < 0.01$, $***P < 0.001$, two-way ANOVA, $n = 8$ –9/group. No significant influence is observed on the locomotor function of rats after spinal application of LV-shSHP2 compared to LV-GFP ($p > 0.05$, two-way ANOVA). (I) Scheme of the experimental procedure. Five days after lentivirions injection, BDNF (at 100 ng) was intrathecally administered twice per day at a 30 min-interval, repeated for 2 days. Both PWT and locomotor function were measured before lentivirions injection, and then on day 1, 3, 5 and 7 after the last BDNF administration, respectively. Electrophysiological recording was performed 7 days after lentivirions injection, and Western blot was carried out on day 7 after behavioral test for the last BDNF injection.



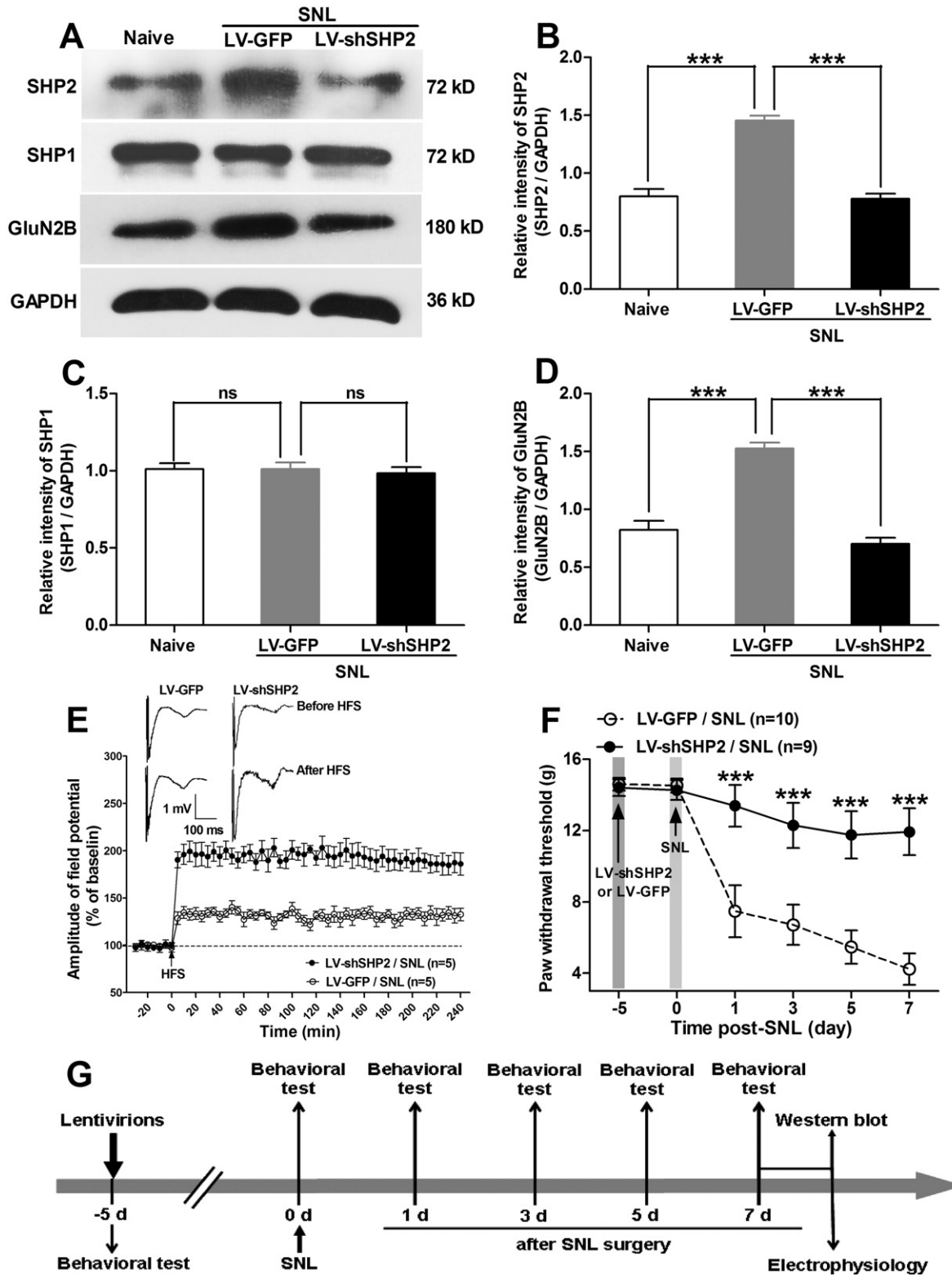


Fig. 10. Knockdown of SHP2 in the spinal cord by intrathecal delivery of LV-shSHP2 prevents nerve injury-evoked GluN2B up-regulation as well as spinal LTP occlusion and pain allodynia development in SNL rats. (A–D) Western blot of SHP2, SHP1, and GluN2B expression in synaptosomal fraction extracted from the spinal dorsal horn tissue in naïve rats, and in SNL rats infected with LV-shSHP2 or LV-GFP. (A) Representative of Western blot bands for SHP2, SHP1, and GluN2B. GAPDH is used as internal control. (B–D) analysis of the relative intensity of SHP2 (B), SHP1 (C) and GluN2B (D). Note that the expression of SHP2 but not SHP1 is statistically decreased in LV-shSHP2 infected rats compared to LV-GFP infected rats. Similarly, intrathecal delivery of LV-shSHP2 significantly inhibits the nerve injury-induced increase of GluN2B expression in SNL rats. *** $P < 0.001$, one-way ANOVA, $n = 4\text{--}5/\text{group}$. (E) Effects of LV-shSHP2 on HFS-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn. Note that the amplitude of C-fiber-evoked field potentials is dramatically increased after HFS of the sciatic nerve in LV-shSHP2-treated rats compared to LV-GFP-treated rats, implying that the occlusion of HFS-induced spinal LTP by SNL surgery is obviously prevented by intrathecal delivery of LV-shSHP2 ($n = 5/\text{group}$). Traces at top are recorded at 10 min before and 3 h after HFS of the sciatic nerve, respectively. (F) Effects of LV-shSHP2 on SNL-evoked pain allodynia. Note the decreased paw withdrawal threshold induced by SNL surgery is significantly restored in LV-shSHP2 infected rats compared to LV-GFP infected rats, suggesting that knockdown of SHP2 by SHP2 siRNA prevents nerve injury-evoked pain allodynia in SNL rats. *** $P < 0.001$, two-way ANOVA, $n = 9\text{--}10/\text{group}$. (G) Scheme of the experimental procedure. Lentivirions was intrathecally delivered to rats on day 5 before SNL surgery. Both PWT and locomotor function were measured before lentivirions injection and SNL operation, and then on day 1, 3, 5 and 7 after SNL surgery, respectively. Electrophysiological recording and Western blot were performed 7 days after SNL surgery and behavioral test.

induction of LTP at C-fiber synapses via activation of GluN2B-NMDA receptors, and this BDNF-mediated LTP-like state in rats suffered from SNL therefore occludes the subsequent development of spinal LTP elicited by tetanic electrical stimulation of the peripheral sensory afferents (Ohnami et al., 2011; Rygh et al., 2000). Secondly, we provide biochemical evidence showing that increased BDNF in the spinal dorsal horn induces a functional up-regulation of GluN2B-NMDA receptors in SNL rats, indicating the activation of GluN2B-NMDA receptors by BDNF following spinal nerve injury. It has been documented that activation of GluN2B-NMDA receptors is particularly crucial for effects of BDNF on long-term synaptic plasticity as well as learning and memory (Bekinschtein et al., 2014; Bramham and Panja, 2014; Edelman et al., 2014; Gomez-Palacio-Schjetnan and Escobar, 2013; Leal et al., 2014; Lu et al., 2008), we therefore suggest that the BDNF-induced functional up-regulation of GluN2B-NMDA receptors in the spinal dorsal horn may underlie the development of BDNF-mediated central sensitization (Biggs et al., 2010; Garraway et al., 2003; Lu et al., 2007; Malcangio and Lessmann, 2003) and persistent pain in neuropathic rats (Geng et al., 2010; Merighi et al., 2008b; Obata and Noguchi, 2006; Ren and Dubner, 2007; Smith, 2014).

There is considerable evidence showing that in the hippocampus, the involvement of GluN2B-NMDA receptors in LTP is dependent on the induction protocol, that is, GluN2B-NMDA receptors are required for LTP induced by the spike-timing protocol, but not by the pairing or HFS protocol (Zhang et al., 2008). However, numerous studies have shown that in the spinal dorsal horn, high-frequency (100 Hz) stimulation of C fibers (as used in our present study) may be most powerful to produce a strong depolarization in superficial dorsal horn neurons, which is sufficient to trigger a strong calcium influx into the postsynaptic cell via NMDA receptor channels and/or voltage-dependent calcium channels for LTP induction (Liu and Sandkuhler, 1995, 1997). Different from the hippocampus, GluN2B-NMDA receptors have been proved to be necessary for the induction of spinal LTP induced by high-frequency stimulation (HFS) of the sciatic nerve at C fibers (Pedersen and Gjerstad, 2008; Qu et al., 2009).

Possible roles of NMDA receptor GluN2A as well as AMPA receptors trafficking in BDNF-mediated spinal LTP induction and neuropathic pain development in SNL rats

Apart from GluN2B, the NMDA receptor subunit GluN2A may be another possible target of BDNF-induced modulation of synaptic plasticity. BDNF enhances the expression and trafficking of GluN2A in cultured cortical and hippocampal neurons, and induces the phosphorylation of GluN2A subunit in PSD of rat spinal cord (Di et al., 2001; Hayashi et al., 2000), which is associated with an increase in NMDA receptor activity (Caldeira et al., 2007b; Small et al., 1998), whereas absence of BDNF induces a reduction of GluN2A level in the visual cortex of BDNF knock-out mice (Margottil and Domenici, 2003). Involvement of GluN2A-containing NMDA (GluN2A-NMDA) receptor in the development of LTP is supported by the findings that pharmacological inhibition of GluN2A by NVP-AAM077 blocks the induction of LTP in multiple brain regions including the hippocampus (Bartlett et al., 2007; Fox et al., 2006; Jin and Feig, 2010; Li et al., 2007; Liu et al., 2004; Volianskis et al., 2013), the cortex (Massey et al., 2004) as well as the amygdala (Dalton et al., 2012; Muller et al., 2009) and the nucleus accumbens (Schotanus and Chergui, 2008). Deletion of GluN2A subunit in mice results in a impaired induction of postsynaptic LTP in the dentate gyrus (Kannagara et al., 2014) and the visual cortex (Philpot et al., 2007), and at the mossy fiber to granule cell synapse in the cerebellum (Andreescu et al., 2011). On the other hand, the GluN2A-NMDA receptor is required for the glycine-evoked membrane insertion of new AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) receptors (AMPA) as well as the induction of LTP in the rat visual cortex (Li and Wang, 2013), and synaptic GluN2A-NMDA receptors are more likely to mediate the transiently increased phosphorylation of

AMPA GluA1 subunit at serine site S831 by bath application of NMDA to hippocampal slices from rats (Ai et al., 2011). In mature cultured hippocampal neurons, GluN2A-NMDA receptors promote the surface expression of GluA1, primarily by regulating its surface insertion (Kim et al., 2005). An increased GluA1 and the transmembrane AMPAR regulatory protein, which is involved in AMPAR trafficking, are found in knock-in (KI) mice in which GluN2B is replaced with GluN2A (Hamada et al., 2014). Using RNA interference (RNAi) against GluN2A or GluN2B, it is found that GluA1 surface delivery is inhibited by knockdown of GluN2A (Kim et al., 2005), while GluN2B knockdown increases synaptic AMPARs (Hall et al., 2007). However, in the anterior cingulate cortex (ACC) of adult mice, it is reported that the NMDA receptor GluN2B subunit is critical for AMPAR GluA1 insertion and LTP induction (Descalzi et al., 2012b; Zhao et al., 2009).

Emerging evidence suggests that AMPAR trafficking and phosphorylation of AMPARs subunit play also important roles in activity-dependent LTP (Malinow, 2003; Yao et al., 2008) and synaptic plasticity (Anggono and Haganir, 2012; Haganir and Nicoll, 2013). For instance, the NMDA receptor-dependent LTP is mediated by the surface insertion and synaptic delivery of GluA1 (Hayashi et al., 2000) or by an increased phosphorylation of GluA1 at S831 (Ai et al., 2011; Lee et al., 2000). The AMPAR subunit GluA1 membrane insertion and GluA2 internalization in dorsal horn neurons of the spinal cord have emerged to participate in the maintenance of NMDA receptor-dependent nociceptive hypersensitivity and central sensitization in persistent inflammatory pain (Kopach et al., 2011, 2013; Park et al., 2008, 2009; Tao, 2010, 2012), and GluA1 phosphorylation contributes to postsynaptic amplification of neuropathic pain in the insular cortex (Qiu et al., 2014). In addition, it is reported that BDNF can up-regulate the expression and phosphorylation of AMPAR subunits in cultured cortical (Nakata and Nakamura, 2007; Narisawa-Saito et al., 1999) and hippocampal neurons (Caldeira et al., 2007a), and induce the delivery of AMPARs to the synapse (Caldeira et al., 2007a). The BDNF-induced AMPAR trafficking and GluA1 phosphorylation are suggested via ERK (extracellular signal-regulated kinase)- or NMDA receptor subunit GluN2B-dependent mechanisms (Li and Keifer, 2009; Wu et al., 2004). Taken together, these findings suggest that the BDNF-mediated AMPAR trafficking or subunit phosphorylation via activation of NMDA receptor subunit GluN2A or GluN2B in the dorsal horn neurons of the spinal cord is probably an alternative contributor to the development of spinal LTP and pain hypersensitivity in rats suffered from nerve injury.

Involvement of SHP2 in BDNF-mediated up-regulation of GluN2B-NMDA receptors as well as spinal LTP induction and neuropathic pain development in SNL rats

Considerable evidence has accumulated that BDNF should bind to and activate TrkB, a receptor tyrosine kinase (RTK), to exert its effects (Park and Poo, 2013). Acting as a major regulator of RTK (Grossmann et al., 2010), SHP2 has been implicated in BDNF/TrkB (Araki et al., 2000; Easton et al., 2006) and GluN2B-NMDA receptors (Lin et al., 1999; Peng et al., 2012) signaling pathway. In our present study, we provide further evidence clarifying that spinal SHP2 is required for BDNF-mediated GluN2B-NMDA receptors activation as well as spinal LTP induction and pain hypersensitivity development in neuropathic rats. We first ascertained the contribution of BDNF to the phosphorylation of SHP2 in the spinal dorsal horn in rats following spinal nerve injury, suggesting that increased BDNF activates dorsal horn SHP2 in SNL rats. Next, using a potent SHP2 protein tyrosine phosphatase inhibitor NSC-87877 (Chen et al., 2006; Peng et al., 2012) or RNA interference (RNAi) against SHP2, we disclosed that either blockade of SHP2 phosphorylation or knockdown of SHP2 in the spinal cord, could inhibit the BDNF-mediated GluN2B-NMDA receptors activation as well as spinal LTP induction and pain allodynia elicitation in intact rats, and reduce the nerve injury-evoked GluN2B-NMDA receptors up-regulation and spinal LTP occlusion, and also alleviate pain allodynia in neuropathic

rats as well, implying that BDNF-induced SHP2 phosphorylation in the spinal dorsal horn is required for subsequent GluN2B-NMDA receptors activation and spinal LTP-like state induction, and ultimately for neuropathic pain development in rats following spinal nerve injury. It has been documented that BDNF enhances association of SHP2 with the NMDA receptor subunit GluN2B in the cortical postsynaptic density (PSD) (Lin et al., 1999), and phosphorylation of spinal SHP2 via signal regulatory protein alpha 1 (SIRP α 1) induces SIRP α 1-SHP2 interaction, which subsequently triggers SHP2/PSD-95/GluN2B signaling, and thereby playing a role in the development of neuropathic pain (Peng et al., 2012). Together these results with our present findings, we suggest that after nerve injury, increased BDNF causes phosphorylation of SHP2 in the spinal dorsal horn, which subsequently triggers SHP2/PSD-95/GluN2B cascade and induces spinal LTP at C-fiber synapses via activation of GluN2B-NMDA receptors, thereby playing a vital role in the development of central sensitization and pain hypersensitivity in neuropathic rats.

It is now well accepted that SHP2 can act upstream of the src family kinases (SFK), or on their regulators, to promote Ras/extracellular signal-regulated protein kinase (ERK)/mitogen-activated protein kinase (MAPK) activation in BDNF/TrkB signaling (Cunnick et al., 2002; Dance et al., 2008; Easton et al., 2006; Kumamaru et al., 2011). Activation of ERK coupled to ERK-dependent phosphorylation of the transcription factor cAMP response element-binding protein (CREB) has been found to be required for the induction of BDNF-induced LTP in intact rat hippocampus (Ying et al., 2002) and the spinal dorsal horn (Zhou et al., 2008). In addition, activation of MAPK/ERK-CREB pathway in the spinal cord has been documented to be necessary for induction and maintenance of spinal LTP induced by tetanic stimulation of the sciatic nerve in intact rats (Xin et al., 2006), and for nerve injury-induced neuropathic pain (Song et al., 2005) as well as cancer-induced bone pain in animal models (Wang et al., 2011). In the anterior cingulate cortex (ACC), accumulative evidence also suggests that the MAPK/ERK-CREB signaling pathway is essential for the induction of cingulate LTP and pain-related negative emotion (Cao et al., 2009a,b; Toyoda et al., 2007). CREB has been known to be a downstream transcription factor of the cAMP and calcium signal transduction pathways, and functions as a positive regulator of memory formation and LTP (Kida, 2012; Kida and Serita, 2014). In the ACC, it is revealed that activation of NMDA receptors and voltage-gated calcium channels may induce an increase in intracellular calcium level (Wei et al., 2006; Zhao et al., 2005). The rise in calcium leads to activation of various intracellular protein kinases and phosphatases (Wei et al., 2003), such as calcium-stimulated adenylyl cyclases (AC1 and 8) and calcium/calmodulin (CaM)-dependent protein kinases (PKC, CaMKII and CaMKIV) (Zhuo, 2006), which subsequently trigger MAPK/ERK-CREB signaling cascade and elicit cAMP response element-mediated gene expression including NMDA receptor subunit GluN2B (Rani et al., 2005; Zhuo, 2009), AMPAR GluA1 (Middei et al., 2013), BDNF (Ou and Gean, 2007; Tabuchi, 2008), etc., thereby resulting in the plastic up-regulation of them in protein synthesis (Kida, 2012; Kida and Serita, 2014). Such possible positive feedback is believed to play a crucial role in the formation of long-lasting plastic change in synaptic transmission within the ACC, and thus contribute to persistent pain (Descalzi et al., 2012a; Zhuo, 2007). In the present study, we demonstrated that phosphorylation of SHP2 in the spinal cord participates in BDNF-mediated up-regulation of GluN2B-NMDA receptors and spinal LTP induction. We here conclude that increased BDNF in the spinal cord may induce activation of MAPK/ERK-CREB signaling pathway through SHP2, subsequently triggers the similar positive feedback as that within the ACC, which is responsible for the up-regulation of GluN2B-NMDA receptors and the maintenance of spinal LTP, and therefore underlies the development of neuropathic pain following peripheral nerve injury.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XD carried out the electrophysiological studies and the biochemical experiments, participated in the design of the study as well as acquired and analyzed data. JC, SL and XDL participated in the behavioral test and performed the statistical analysis. YW participated in the design of the study. GGX contributed to the conception and design of the study, participated in the statistical analysis of data, drafted the manuscript and made final approval of the version to be submitted. All authors have read and approved the final manuscript.

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