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ORIGINAL PAPER

BDNF Contributes to Spinal Long-Term Potentiation and Mechanical Hypersensitivity Via Fyn-Mediated Phosphorylation of NMDA Receptor GluN2B Subunit at Tyrosine 1472 in Rats Following Spinal Nerve Ligation

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Abstract Previously we have demonstrated that brain-derived neurotrophic factor (BDNF) contributes to spinal long-term potentiation (LTP) and pain hypersensitivity through activation of GluN2B-containing *N*-methyl-D-aspartate (GluN2B-NMDA) receptors in rats following spinal nerve ligation (SNL). However, the molecular mechanisms by which BDNF impacts upon GluN2B-NMDA receptors and spinal LTP still remain unclear. In this study, we first documented that Fyn kinase-mediated phosphorylation of GluN2B subunit at tyrosine 1472 (pGluN2B^{Y1472}) was involved in BDNF-induced spinal LTP and pain hypersensitivity in intact rats. Second, we revealed a co-localization of Fyn and GluN2B-NMDA receptor in cultured dorsal horn neurons, implying that Fyn is a possible intermediate kinase linking BDNF/TrkB signaling with GluN2B-NMDA receptors in the spinal dorsal horn. Furthermore, we discovered that both SNL surgery and intrathecal active Fyn could induce an increased expression of dorsal horn pGluN2B^{Y1472}, as well as pain hypersensitivity in response to von Frey filaments stimuli; and more importantly, all these actions were effectively abrogated by pre-treatment with either PP2 or ifenprodil to respectively inhibit Fyn kinase and GluN2B-NMDA receptors activity. Moreover, we found that intrathecal administration of BDNF scavenger TrkB-Fc prior to SNL surgery, could prevent the nerve injury-induced increase of both pFyn^{Y420} and pGluN2B^{Y1472} expression, and also inhibit the mechanical allodynia in neuropathic rats. Collectively, these results

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suggest that Fyn kinase-mediated pGluN2B^{Y1472} is critical for BDNF-induced spinal LTP and pain hypersensitivity in SNL rats. Therefore, the BDNF-Fyn-GluN2B signaling cascade in the spinal dorsal horn may constitute a key mechanism underlying central sensitization and neuropathic pain development after peripheral nerve injury.

Keywords Brain-derived neurotrophic factor · Fyn · GluN2B-containing NMDA receptor · Long-term potentiation · Neuropathic pain · Spinal dorsal horn

Introduction

The mechanisms underlying neuropathic pain development still remain largely unknown. Emerging evidence suggests that sensory information processing in the spinal dorsal horn appears to undergo significant plastic changes e.g. LTP in synaptic efficacy following peripheral nerve injury or inflammation [1–4], which is considered as a potential mechanism underlying central sensitization and post-injury pain hypersensitivity [5–9]. Previously we have demonstrated that BDNF contributes to the induction of spinal LTP and pain hypersensitivity through activation of GluN2B-containing *N*-methyl-D-aspartate (GluN2B-NMDA) receptors in rats following spinal nerve ligation (SNL) [10–12]. Yet, the molecular mechanisms by which BDNF impacts upon GluN2B-NMDA receptors and spinal LTP are unclear.

As an important member of the Src-family tyrosine kinases (SFKs), Fyn kinase is critical for synaptic plasticity and many pathophysiological processes [13–17]. Phosphorylation of GluN2B subunit by Fyn is one of the major regulatory mechanisms for NMDA receptors function [15, 18]. Tyrosine 1472 (Tyr1472) of GluN2B is the specific site of Fyn-mediated phosphorylation and phosphorylation of this site plays a crucial role in the induction of hippocampal LTP and hippocampus-dependent memory formation [19, 20]. Activation of Fyn kinase in dorsal hippocampus is essential for contextual fear conditioning [21] and up-regulation of GluN2B phosphorylation at Tyr1472 (pGluN2B^{Y1472}) in mice with neuronal Fyn overexpression is implicated in the pathogenesis of brain injury following neonatal hypoxia–ischemia [22, 23]. In pain-related dorsal horn of the spinal cord, altered Fyn activity, through modulating NMDA receptor function, plays an important role in the sustained pain sensitization [24]. GluN2B phosphorylation at Tyr1472 in the spinal dorsal horn contributes to NMDA-induced pain hypersensitivity in mice [25]; and also, Fyn kinase-mediated pGluN2B^{Y1472} is involved in the development of neuropathic pain in animal models of peripheral nerve injury [26–28] or diabetic neuropathy [29].

In the other hand, BDNF is implicated as an endogenous ligand that regulates NMDA receptor-dependent synaptic plasticity [30–33]. In the hippocampus, acting through the trkB receptor, BDNF elicits phosphorylation-dependent enhancement of NMDA receptors activity [34]. Also, BDNF acutely increases tyrosine phosphorylation of the GluN2B-NMDA receptors in cortical and hippocampal postsynaptic densities [35]. Blockade of GluN2B-NMDA receptors prevents BDNF-induced enhancement of glutamatergic transmission in hippocampal neurons [36]. It is accepted that Fyn is an intermediate kinase linking BDNF/TrkB signaling with GluN2B-NMDA receptors in the process of learning and memory [30, 31, 37, 38]. In the hippocampus, Xu and colleagues [39] demonstrate that BDNF rapidly increases NMDA receptor activity through Fyn-mediated pGluN2B^{Y1472}, which may constitute a key mechanism underlying BDNF-mediated synaptic plasticity. More recently, Hildebrand et al. [40] show that BDNF mediates NMDA receptor potentiation through phosphorylation of GluN2B by Fyn activation in dorsal horn lamina I neurons in acute spinal slices from adult rats. These findings raise a possibility that the BDNF-Fyn-GluN2B signaling pathway in the dorsal horn may be involved in BDNF-mediated spinal LTP and pain hypersensitivity following peripheral nerve injury.

In this study, we present evidence to show that Fyn kinase-mediated pGluN2B^{Y1472} is required for BDNF-induced spinal LTP and mechanical hypersensitivity during the pathogenesis of neuropathic pain in rats subjected to SNL surgery.

Materials and Methods

Chemicals, Antibodies, and Animals

Chemicals: BDNF (Sigma-Aldrich, St. Louis, MO) was first dissolved as a concentrated stock solution (5.0 μg/ml) in 0.9% sterile saline. 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. Recombinant active Fyn protein (RayBiotech, Norcross, GA) was stored at −80 °C and diluted to the working concentration of 0.1 μg/μl with 0.9% saline. Fyn39-57 and scrambled Fyn39-57 interfering peptides as well as their TAT-linked versions (TAT-Fyn39-57 and TAT-scrambled Fyn39-57) were synthesized by GL Biochem (Shanghai, China), dissolved in 0.9% sterile saline as a 3.75 μg/μl store solution and stored at −20 °C. Peptides were synthesized with the following sequences as described in previous studies [40, 41]: Fyn39-57 (YPSFGVTSIPNYN-NFHAAG, Fyn amino acids 39-57), scrambled Fyn39-57

(PSAYGNPGSAYFNFTNVHI). Both of Fyn39-57 and scrambled Fyn39-57 were linked to TAT transduction domains (RKKRRQRRR), which allowed us to apply the membrane permeant form of the selective Fyn interfering peptide extracellularly to the dorsal horn neurons. Ifenprodil (Sigma) as well as PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine) and its inactive analogue PP3 (4-amino-7-phenylpyrazolo[3,4-d]pyrimidine) (Merck Millipore, Darmstadt, Germany) 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%.

Antibodies

Polyclonal mouse anti-GluN2B, rabbit anti-Glu2A, rabbit anti-Src as well as monoclonal rabbit anti-pSrc^{Y416} antibodies that detects phosphorylated amount of Tyr420 in Fyn [40, 42]. were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal rabbit anti-Fyn and rabbit anti-pGluN2B^{Y1472} antibodies were obtained from Abcam (Cambridge, MA) and Merck Millipore (Darmstadt, Germany), respectively. Monoclonal mouse anti-beta-tubulin isotype III antibody was obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and horseradish peroxidase–labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 568-conjugated goat anti-mouse IgG and Alexa Fluor 488/Cy3-conjugated goat anti-rabbit IgG were obtained from Invitrogen (Carlsbad, CA).

Animals

Male Sprague-Dawley rats weighing 150–180 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 [43] and were approved by the Animal Care and Use Committee of Peking University.

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 [44]. 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. [45]. Briefly, a PE-10 polyethylene catheter was implanted between the L5 and L6 vertebrae to reach the lumber 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-µl volume of solution followed by 10-µl 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.

Behavioral Studies

All behavioral experiments were performed blinded to the treatment group.

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 [10, 46]. The 50% PWT in response to a series of von Frey filaments (Stoelting, Wood Dale, IL) was determined by the Up and Down method [47]. The rat was placed on a metal mesh floor covered with an inverted clear plastic cage (18 × 8 × 8 cm) and allowed a 20-min 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 2–3 s. 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 six stimuli after the first change in response had been observed. The 50% PWT was calculated using the following formula: $50\%\ \mathrm{PWT}\,(g) = (10^{[X_f + k\delta]})/10,000$, 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 [48]. 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) [49].

Measurement of Drug Effects

The first behavioral experiment was performed to examine whether Fyn kinase-mediated activation of GluN2B-NMDA receptors is involved in BDNF-induced pain hypersensitivity in intact rats (see Fig. 1). To confirm the long-lasting effect of BDNF on mechanical allodynia development as reported in our previous study [10], BDNF at 100 ng was intrathecally administrated to rats twice per day at a 30 min-interval, continued for 2 days, and the 50% PWT was measured on day 3 after the last drug injection on the second day. To further determine whether Fyn kinase and GluN2B-NMDA receptor were involved in BDNF-induced pain hypersensitivity, effect of pre-treatment with TrkB-Fc, PP2, or ifenprodil on BDNF action was then investigated, respectively. Here, TrkB-Fc, a TrkB-immunoglobulin G fusion protein is often applied to neutralize BDNF effect [10, 50]; PP2, a Src-family tyrosine kinase inhibitor, is usually used as a pharmacological inhibitor for specifically inhibiting Fyn kinase activity [39, 51, 52]; and ifenprodil is well known as a selective GluN2B-NMDA receptor inhibitor [10, 12]. According to the methods described in previous studies [10, 12, 53], TrkB-Fc (25 ng/μl, 20 μl), PP2 (1.5 μg/μl, 20 μl), or ifenprodil (1.0 μg/μl, 20 μl) was intrathecally delivered to rats twice per day at 30 min prior to BDNF administration. An equal dose of IgG (25 ng/μl, 20 μl), PP3 (1.5 μg/μl, 20 μl, an inactive analogue of PP2), or DMSO was applied in the same manner as the control of TrkB-Fc, PP2 and ifenprodil, respectively. 50% PWT to von Frey filaments was then measured on the day before drug application, and then on day 3 after the last BDNF injection. Alterations of Fyn, Src, GluN2A, GluN2B as well as $pFyn^{Y420}$ and $pGluN2B^{Y1472}$ in the spinal dorsal horn were assessed after finishing behavioral test.

In order to prove that Fyn kinase is a linker between BDNF and GluN2B-NMDA receptors for spinal LTP and allodynia, we performed additional experiments to examine whether pre-treatment with interfering peptide TAT-Fyn39-57, that can disrupt the upregulation of NMDAR currents by Fyn kinase [40, 41] prior to the intrathecal injection of BDNF, could prevent the BDNF-induced spinal LTP and pain hypersensitivity in intact rats (see Fig. 2). While BDNF (100 ng) was intrathecally administrated to rats twice per day at a 30 min-interval (continued for 2 days), TAT-Fyn39-57 (3.75 μg/μl, 20 μl) or an equal dose of TAT-scrambled Fyn39-57 was intrathecally delivered to rats twice per day at 30 min prior to BDNF administration. The paw withdrawal threshold in rats was measured on the day before drug application, and then on day 3 after the last BDNF injection. Expression of Fyn, $pFyn^{Y420}$, GluN2B and $pGluN2B^{Y1472}$ in the spinal dorsal horn were detected on day 3 after drug injection and behavioral assessment.

Furthermore, we determined whether intrathecal injection of TrkB-FC prior to SNL surgery could prevent nerve injury-induced increase of $pFyn^{Y420}$ and $pGluN2B^{Y1472}$, and abrogate the pain hypersensitivity in neuropathic rats (see Fig. 4). TrkB-Fc (25 ng/μl, 20 μl), 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. Ipsilateral PWT to von Frey filaments was measured before drug injection as well as after the last drug injection on day 14 post-surgery. Alterations of dorsal horn Fyn, $pFyn^{Y420}$ as well as GluN2B and $pGluN2B^{Y1472}$ were detected after finishing behavioral test on day 14 post-surgery.

We also performed behavioral experiment to examine whether pre-treatment with the SFKs inhibitor PP2, or GluN2B-NMDA receptor inhibitor ifenprodil, could alleviate pain hypersensitivity in neuropathic rats (see Fig. 5c). PP2 (1.5 μg/μl, 20 μl) or ifenprodil (1.0 μg/μl, 20 μl) 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. An equal dose of PP3 (1.5 μg/μl, 20 μl) or DMSO was applied in the same manner as the control of PP2 and ifenprodil, respectively. Ipsilateral PWT to von Frey filaments was then measured before SNL and on day 14 after SNL surgery.

In addition, we investigated whether recombinant active Fyn kinase could produce pain hypersensitivity in intact rats, and whether pre-treatment with PP2 or ifenprodil could inhibit the Fyn kinase action (see Fig. 5d). To test effect of Fyn kinase on pain hypersensitivity in intact rats, recombinant active Fyn (0.1 μg/μl, 20 μl), which has been widely applied to examine Fyn kinase action in previous studies [51, 54], was intrathecally administrated to rats twice per day at a 12 h-interval (continued for 2 days), and the 50% PWT in rats was measured on day 3 after the last drug injection on the second day. To further determine whether pre-treatment with PP2 or ifenprodil could inhibit active Fyn-induced pain hypersensitivity, PP2 (1.5 μg/μl, 20 μl) or ifenprodil (1.0 μg/μl, 20 μl) was intrathecally delivered to rats twice per day at 30 min prior to recombinant active Fyn administration. An equal dose of PP3

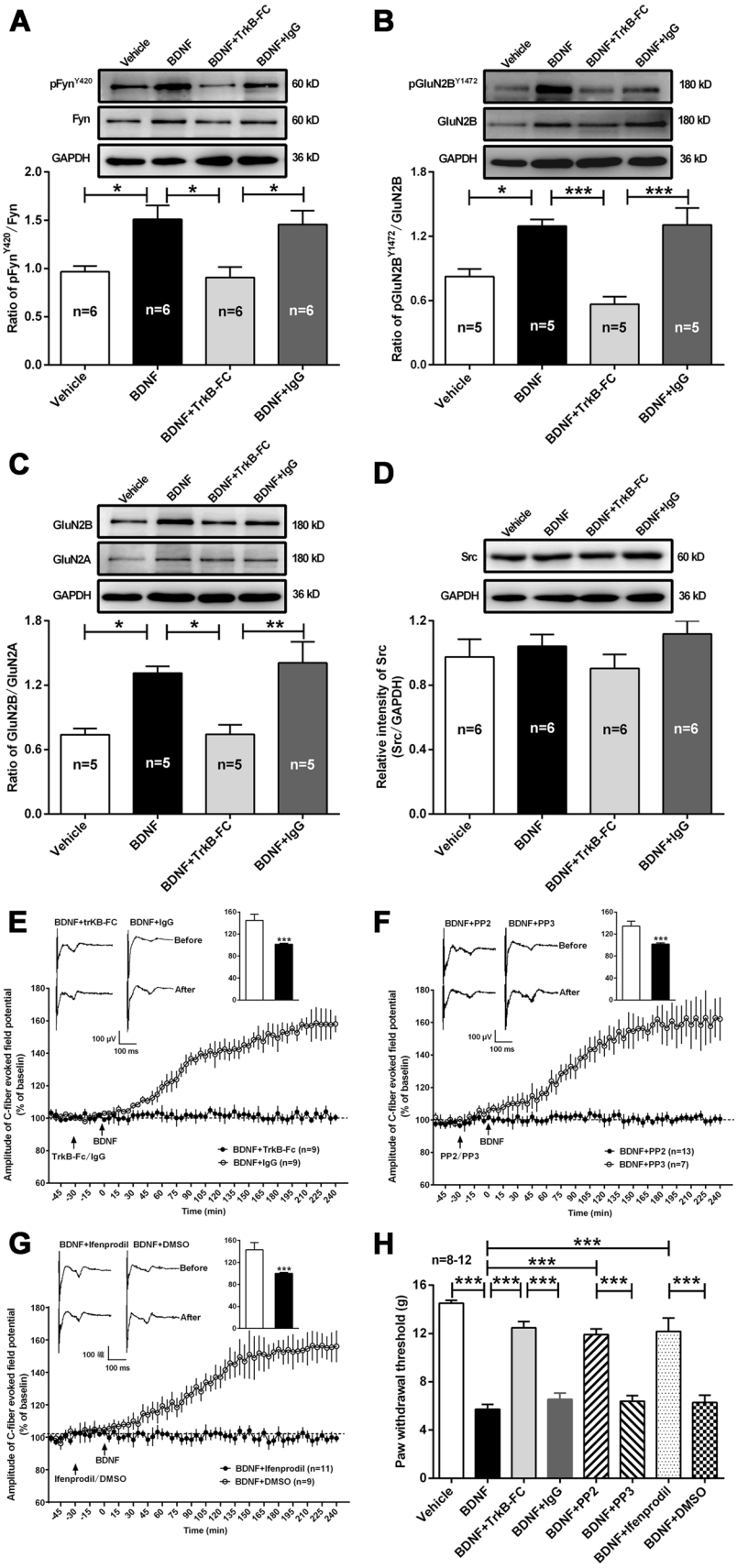


Fig. 1 Effects of spinal BDNF on the activation of dorsal horn Fyn and GluN2B-NMDA receptors, as well as on the induction of spinal LTP and pain hypersensitivity in intact rats. **a**–**d** Western blot of phosphorylated Fyn at tyrosine 420 ($pFyn^{Y420}$) (**a**), phosphorylated GluN2B at tyrosine 1472 ($pGluN2B^{Y1472}$) (**b**), as well as GluN2B, GluN2A (**c**) and Src (**d**) expression in L4 and L5 spinal dorsal horn. *Upper* representative of Western blot bands; *lower* summary of statistical analysis. GAPDH is used as an internal control. Note that the ratios of $pFyn^{Y420}$ to Fyn, $pGluN2B^{Y1472}$ to GluN2B, and GluN2B to GluN2A are increased in rats received intrathecal BDNF but not vehicle injection, and all these BDNF effects can be significantly abrogated by pre-treatment with TrkB-FC to inhibit the BDNF/TrkB signaling. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA, n = 5–6 per group. **e**–**g** Effects of TrkB-Fc, PP2 or ifenprodil on BDNF-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn. Note that perfusion of either TrkB-FC (25 ng/μ) (**e**), PP2 (1.5 μg/μl) (**f**), or ifenprodil (1.0 μg/μl) (**g**) for 30 min before spinal application of BDNF (100 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. The *bar graph* indicates the mean amplitude of C-fiber-evoked field potentials during the 1–4 h after BDNF application in each corresponding group. $^{***}p < 0.001$, two-tailed unpaired *t* test, n = 7–13 per group. **f** Effects of TrkB-FC, PP2, as well as ifenprodil on BDNF-evoked pain hypersensitivity. Note that pre-treatment with TrkB-FC, PP2, or ifenprodil can significantly block the BDNF-induced decrease of paw withdrawal threshold in response to von Frey filaments stimuli. $^{***}p < 0.001$, two-way ANOVA, n = 8–12 per group

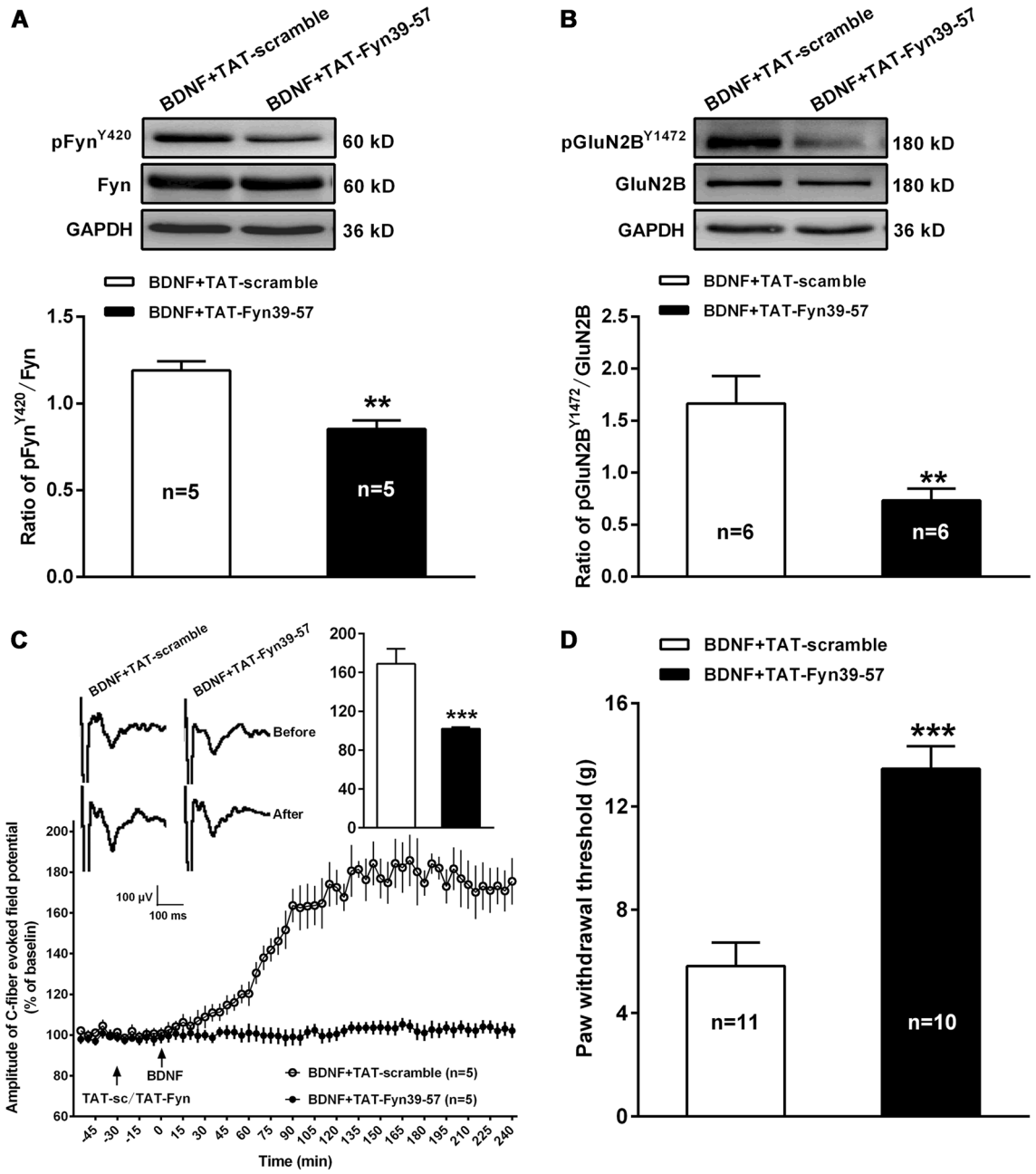


Fig. 2 Effects of pre-treatment with interfering peptide TAT-Fyn39-57 on BDNF-induced phosphorylation of Fyn at tyrosine 420 ($pFyn^{Y420}$) and GluN2B at tyrosine 1472 ($pGluN2B^{Y1472}$), as well as on BDNF-induced spinal LTP and pain hypersensitivity in intact rats. **a, b** Western blot of $pFyn^{Y420}$ (**a**) and $pGluN2B^{Y1472}$ (**b**) expression in L4 and L5 spinal dorsal horn. *Upper* representative of Western blot bands; *lower* summary of statistical analysis. GAPDH is used as an internal control. Note that the ratios of $pFyn^{Y420}$ to Fyn and $pGluN2B^{Y1472}$ to GluN2B are significantly decreased in TAT-Fyn39-57-treated rats compared with the control TAT-scramble-treated rats. $^{**}p < 0.01$, two-tailed unpaired *t* test, n = 5–6 per group. **c** Effects of TAT-Fyn39-57 on BDNF-induced LTP of C-fiber-evoked field potentials in the spinal dorsal horn. Note that perfusion of TAT-Fyn39-57 (3.75 μg/μl) for 30 min before spinal application of BDNF (100 ng) can effectively prevent 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. The bar graph indicates the mean amplitude of C-fiber-evoked field potentials during the 1–4 h after BDNF application. $^{***}p < 0.001$, two-tailed unpaired *t* test, n = 5 per group. **d** Effects of TAT-Fyn39-57 on BDNF-evoked pain hypersensitivity. Note that the decreased paw withdrawal threshold induced by BDNF is remarkably restored in TAT-Fyn39-57-treated rats (n = 10) compared with the control TAT-scramble-treated rats (n = 11). $^{***}p < 0.001$, two-tailed unpaired *t* test

(1.5 μg/μl, 20 μl) or DMSO was applied in the same manner as the control of PP2 and ifenprodil, respectively. 50% PWT to von Frey filaments was then measured on the day before drug application, and then on day 3 after the last active Fyn injection.

Also, we examined whether pre-treatment with the above used antagonists or interfering peptides had any effect on baseline paw withdrawal threshold in intact rats (see Supplementary Figure S1). TrkB-Fc (25 ng/μl, 20 μl), PP2 (1.5 μg/μl, 20 μl), TAT-Fyn39-57 (3.75 μg/μl, 20 μl) or ifenprodil (1.0 μg/μl, 20 μl) was intrathecally delivered to rats twice per day at a 12 h-interval, continued for 2 days. The respective equal dose of IgG, PP3, TAT-scrambled Fyn39-57 or DMSO was applied to rats in the same manner as the control of above drugs. 50% PWT to von Frey filaments was measured on the day before drug application, and then at 30 and 60 min after drug injection, respectively.

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 s without falling was determined according to the method reported by Rivlin and Tator [55]. In this study, inclined-plate test was performed for all behavioral experiments in which the rats subjected to intrathecal drugs.

Western Blot

Under deep anesthesia with 10% chloral hydrated (0.3 g/kg, i.p.), the rat lumbar spinal dorsal horn around L4–L5 segment was removed and immediately homogenized in ice-chilled lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP40 (Sigma-Aldrich, Saint Louis, MO, USA), 0.5% sodium deoxycholate (Sigma-Aldrich, Saint Louis, MO), 0.1% sodium docecylsulfate (SDS) and protease inhibitor cocktail (Roche, Indianapolis, Indiana, USA). The homogenates were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was analyzed. The concentration of protein was measured with a BCA assay kit (Pierce, Rockford, IL, USA), and equal amount of protein samples (60 μg) 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: rabbit anti-Fyn (1:1000, Abcam), rabbit anti-pSrc^{Y416} (1:1000, Cell Signaling Technology, for detecting phosphorylated amount of Tyr420 in Fyn [40, 42]), mouse anti-GluN2B (1:1000, Cell Signaling Technology), rabbit anti-pGluN2B^{Y1472} (1:800, Abcam), as well as rabbit anti-Glu2A (1:1000, Cell Signaling Technology), rabbit anti-Src (1:1000, Cell Signaling Technology), 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 Src, Fyn, pFyn^{Y420}, GluN2A, GluN2B, or pGluN2B^{Y1472}to GAPDH band density was used to calculate the alteration of these proteins expression. The ratios of phosphorylated pGluN2B^{Y1472} to total GluN2B as well as pFyn^{Y420} to total Fyn were performed to assess the activated pGluN2B^{Y1472} and pFyn^{Y420}. Western blot assay was performed after behavioral assessment in each group.

Cell Culture and Immunocytochemistry

Primary cultures of spinal cord superficial dorsal horn neurons were prepared from postnatal day 3 rat pups as described previously [56]. Briefly, after decapitation under deep isoflurane anesthesia, a laminectomy was performed and the dorsal third of the spinal cord was cut with razor blade. The superficial dorsal horn strips were incubated for 35 min at 37 °C in Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) (in mM: 137 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 4.2 $NaHCO_3$, 0.3 $Na2HPO_4$, and 5.6 glucose) containing papain (20 U/ml, Sigma). The enzymatic digestion was stopped by adding 3 ml HBSS containing bovine serum albumin (BSA, 1 mg/ml, Sigma), trypsine inhibitor (10 mg/ml, Sigma), and DNAse (0.01%, Sigma). After trituration with a polished Pasteur pipette, the homogenate was centrifugated at 800 rpm for 2 min. The re-suspended cells replaced with Neurobasal growth medium containing B27 supplement, 0.5 mM L-glutamax (Sigma-Aldrich), penicillin and streptomycin (100 IU/ml for each, Gibco), transferrin (10 mg/ml, Sigma), insulin (5 mg/ml, Sigma), putrescine (100 nM, Sigma), and progesterone (20 nM, Sigma), plated on 35-mm dishes coated with poly-D-lysine (0.5 mg/mL, Sigma-Aldrich), and cultured in humidified air with 5% CO_2 at 37 °C for 5 days before use.

Cultured cells were fixed with 4% paraformaldehyde/phosphate buffer (PB) solution for 15 min at 4 °C, rinsed with 0.1 M phosphate-buffered saline (PBS) three times

for 5 min each, and were then blocked in 3% normal goat serum (Sigma) and 0.02% Triton X-100 in 0.1 M PBS (3% NGST) for 1 h. All primary antibodies were diluted in 3% NGST. For double staining of Fyn and GluN2B, cells were incubated in a mixture of rabbit anti-Fyn (1:1000, Upstate Biotechnology) and rabbit anti-GluN2B (1:1000, Cell Signaling) primary antibodies at 4 °C overnight. After rinsed with 0.1 M PBS three times for 5 min each, cells were then incubated in a mixture of Alexa-488-conjugated goat anti-rabbit IgG (1:2000, Invitrogen) and Cy3-conjugated goat anti-rabbit IgG (1:1000, Invitrogen) secondary antibodies for 1 h at room temperature. For double staining of Fyn and beta-tubulin, the same procedure was performed except that cells were incubated in a mixture of rabbit anti-Fyn (1:1000, Upstate Biotechnology) and mouse anti-beta-tubulin isotype III (1:1000, Sigma) primary antibodies and then in a mixture of Alexa-488-conjugated goat anti-rabbit IgG (1:2000, Invitrogen) and Alexa Fluor 568-conjugated goat anti-mouse IgG (1:2000, Invitrogen) secondary antibodies. The cells were rinsed in PBS and observed under a confocal laser-scanning microscope (Olympus, Tokyo, Japan).

In Vivo Electrophysiology

Surgical Preparation

The rat was initially anesthetized with urethane (1.2–1.5 g/kg, i.p.), and then a cannula was inserted into the trachea for artificial respiration and to measure end-tidal CO_2 levels. A catheter was placed in the right jugular vein for continuous administration of anesthetic and for fluid support with Tyrode's solution containing (in mM) NaCl 137, KCl 2.7, $CaCl_2$ 1.4, $MgCl_2$ 1.0, $NaHCO_3$ 6.0, NaH_2PO_4 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 [10, 57]. 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 CO_2 (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-µl volume of solution.

Field Potential Recording and LTP Induction

The C-fiber-evoked field potentials were recorded at a depth of 100–500 µm from the dorsal surface of L4–L5 spinal cord with 1–3 MΩ parylene-coated tungsten microelectrodes (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 studies [4, 10]. 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%).

For BDNF-induced LTP: after six stable control field potentials were recorded, BDNF at 100 ng (in 20-µl 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 as described above (0.5-ms duration, 10–20 V, delivered at 5-min intervals) were measured at 5-min intervals for up to 240 min. To test the pharmacological effect of some inhibitors on BDNF-induced LTP in intact rats, TrkB-Fc (25 ng/µl, 20 µl), PP2 (1.5 µg/µl, 20 µl), TAT-Fyn39-57 (3.75 µg/ul, 20 µl), or ifenprodil (1.0 µg/µl, 20 µl) was spinally administrated 30 min before BDNF (100 ng) application. An equal dose of IgG (25 ng/µl, 20 µl), PP3 (1.5 µg/µl, 20 µl, an inactive analogue of PP2), TAT-scrambled Fyn39-57 (3.75 µg/ul, 20 µl), or DMSO was applied in the same manner as the control of TrkB-Fc, PP2, TAT-Fyn39-57 and ifenprodil, respectively (see Figs. 1e–g, 2c).

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.

Additionally, effects of the above used drugs on baseline C-fiber-evoked field potentials also were examined in intact rats (see Supplementary Figure S1A–D). After six stable control field potentials were recorded, TrkB-Fc (25 ng/μl, 20 μl), PP2 (1.5 μg/μl, 20 μl), TAT-Fyn39-57 (3.75 μg/μl, 20 μl) or ifenprodil (1.0 μg/μl, 20 μl) were applied topically to the dorsal surface of the spinal cord. An equal dose of IgG (25 ng/μl, 20 μl), PP3 (1.5 μg/μl, 20 μl), TAT-scrambled Fyn (3.75 μg/μl, 20 μl) or DMSO was applied in the same manner as the control of TrkB-Fc, PP2, TAT-Fyn39-57 and ifenprodil, respectively. The post-drug field potentials evoked by the same test stimulus as described above (0.5-ms duration, 10–20 V, delivered at 5-min intervals) were measured at 5-min intervals for up to 120 min.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 6 for Windows (GraphPad Software, Inc., La Jolla, CA). All data were expressed as mean ± 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. Differences with $p < 0.05$ were considered statistically significant.

Results

Fyn Kinase-Mediated Phosphorylation of GluN2B Subunit at Tyr1472 is Involved in BDNF-Induced Spinal LTP and Pain Hypersensitivity in Intact Rats

In previous studies, we have reported that BDNF/TrkB signaling in the spinal cord is involved in the development of neuropathic pain through activation of dorsal horn GluN2B-NMDA receptors after peripheral nerve injury [10, 11]. To determine whether Fyn is an intermediate kinase linking BDNF/TrkB signaling with GluN2B-NMDA receptors as seen in the process of learning and memory [39, 58, 59], we first examined effects of spinal BDNF on the expression of dorsal horn activating Fyn in intact rats. It has been documented that phosphorylation of two tyrosine residues regulate the activity of Fyn kinase, with phosphorylation of Tyr420 activating Fyn (Tyr416 in Src) and phosphorylation of Tyr531 inhibiting Fyn (Tyr527 in Src) [18, 60]. We used anti-phospho-Src family (Tyr416) antibody (anti-pSrc^{Y416}), which can detect phosphorylated amount of Tyr420 in Fyn (pFyn^{Y420}) as proved in previous studies [40, 42], to assess the activating Fyn in the spinal dorsal horn. BDNF was intrathecally delivered to intact rats as described in Methods "Measurement of drug effects" subsection, and Western blot analysis was carried out on day 3 after drug injection and behavioral test. As shown in Fig. 1a, spinal application of BDNF induced a significant increase in Fyn phosphorylation at Tyr420 (pFyn^{Y420}), and this increase was effectively inhibited by TrkB-Fc, which is often applied to neutralize BDNF effect [10, 50]. The ratio of pFyn^{Y420} to total Fyn was statistically increased in BDNF-treated rats (1.51 ± 0.14) as compared with vehicle-treated rats (0.97 ± 0.06) ($p < 0.05$, one-way ANOVA, $n = 6$ per group). Pre-treatment with TrkB-Fc (25 ng/μl, 20 μl) significantly inhibited the BDNF-induced elevation of pFyn^{Y420}/total Fyn ratio compared to the control IgG (0.91 ± 0.11 BDNF + TrkB-FC vs. 1.46 ± 0.14 BDNF + IgG) ($p < 0.05$, one-way ANOVA, $n = 6$ per group).

Next, we explored whether spinal BDNF could induce phosphorylation of GluN2B subunit at Tyr1472 via activation of Fyn kinase. As shown in Fig. 1b, intrathecal administration of BDNF to rats produced a significant increase in expression of phosphorylated GluN2B subunit at Tyr1472 (pGluN2B^{Y1472}) in the spinal cord dorsal horn (ratio of pGluN2B^{Y1472} to GluN2B, 1.29 ± 0.06 BDNF vs. 0.82 ± 0.07 vehicle) ($p < 0.05$, one-way ANOVA, $n = 5$ per group). Likewise, the increased expression of dorsal horn pGluN2B^{Y1472}/GluN2B ratio induced by BDNF was effectively blocked in rats pre-treated with TrkB-FC (25 ng/μl, 20 μl) to inhibit BDNF/Trk-B signaling. The ratio of pGluN2B^{Y1472} to GluN2B was statistically decreased in BDNF + TrkB-FC group (0.57 ± 0.07) as compared with the control BDNF + IgG group (1.31 ± 0.16) ($p < 0.001$, one-way ANOVA, $n = 5$ per group). Moreover, we also examined effects of BDNF on dorsal horn GluN2A, GluN2B as well as Src expression in intact rats. We observed a significant increase in GluN2B and the ratio of GluN2B to GluN2A but not GluN2A and Src in rats received intrathecal BDNF; and also, pre-treatment with TrkB-FC could inhibit the increased ratio of GluN2B to GluN2A after BDNF administration (ratio of GluN2B to GluN2A, 1.31 ± 0.06 BDNF versus 0.74 ± 0.05 vehicle, $p < 0.05$; 0.74 ± 0.09 BDNF + TrkB-FC vs. 1.41 ± 0.19 BDNF + IgG, $p < 0.01$) (one-way ANOVA, $n = 5$ per group, Fig. 1c–d).

Furthermore, we investigated the role for Fyn kinase-mediated activation of GluN2B-NMDA receptors in BDNF-induced spinal LTP in intact rats. We found that spinal application of BDNF (100 ng in a 20-μl volume) induced a significant LTP of C-fiber-evoked field potentials in the dorsal horn of the spinal cord, which was almost completely

blocked by the pre-treatment with TrkB-FC (25 ng/μl, 20 μl), PP2 (1.5 μg/μl, 20 μl), or ifenprodil (1.0 μg/μl, 20 μl) 30 min prior to BDNF administration. When compared to the baseline responses (averaged at 30 min prior to drug), the mean C-fiber-evoked field potentials 1–4 h after BDNF application were 144.7 ± 11.3% in BDNF + IgG treated rats and 101.5 ± 1.6% in BDNF + TrkB-FC treated rats ($p < 0.001$, vs. BDNF + IgG group, n = 9 per group, Fig. 1e), as well as 134.4 ± 8.7% in BDNF + PP3 treated rats (n = 7) and 103.6 ± 2.3% in BDNF + PP2 treated rats (n = 13) ($P < 0.001$, vs. BDNF + PP3 group, Fig. 1f), and 143.0 ± 13.1% in BDNF + DMSO treated rats (n = 9) and 100.3 ± 1.9% in BDNF + Ifenprodil treated rats (n = 11) ($P < 0.001$, vs. BDNF + DMSO group, Fig. 1g), respectively (two-tailed unpaired *t* test).

In addition, we assessed the involvement of Fyn kinase-mediated activation of GluN2B-NMDA receptors in BDNF-induced pain hypersensitivity in intact rats. The results showed that intrathecal administration of BDNF to rats indeed produced a mechanical hypersensitivity as assessed by measuring PWTand this BDNF action was significantly inhibited by the pre-treatment with TrkB-FC (25 ng/μl, 20 μl), PP2 (1.5 μg/μl, 20 μl), or ifenprodil (1.0 μg/μl, 20 μl) twice per day at 30 min prior to BDNF administration. As shown in Fig. 1h, the PWT (in gram) was remarkably decreased in BDNF-treated rats (5.72 ± 0.40) in contrast to vehicle-treated rats (14.51 ± 0.24) ($p < 0.001$, one-way ANOVA, n = 11 per group). The decreased PWT induced by spinal BDNF was significantly restored by the pre-treatment of TrkB-FC (12.47 ± 0.54), PP2 (11.91 ± 0.46), or ifenprodil (12.18 ± 1.11) as compared with their respective control of IgG (6.54 ± 0.53), PP3 (6.38 ± 0.46) and DMSO (6.28 ± 0.62) ($p < 0.001$, one-way ANOVA, n = 8–12 per group).

In order to prove that Fyn kinase is a linker between BDNF and GluN2B-NMDA receptors for spinal LTP and allodynia, we examined whether pre-treatment with interfering peptide TAT-Fyn39-57, that can disrupt the upregulation of NMDAR currents by Fyn kinase [40, 41] prior to the intrathecal injection of BDNF, could prevent the BDNF-induced spinal LTP and pain hypersensitivity in intact rats. As our expectation, both of the increased $pFyn^{Y420}$ and $pGluN2B^{Y1472}$, the BDNF-induced LTP as well as the BDNF-induced allodynia were reversed by pre-treatment with interfering peptide TAT-Fyn39-57. The ratios of $pFyn^{Y420}$ to Fyn as well as $pGluN2B^{Y1472}$ to GluN2B were decreased from 1.19 ± 0.05 of TAT-scramble to 0.85 ± 0.05 of TAT-Fyn39-57 ($p < 0.01$, n = 5 per group) and from 1.67 ± 0.26 of TAT-scramble to 0.74 ± 0.11 of TAT-Fyn39-57 ($p < 0.01$, n = 6 per group) (two-tailed unpaired *t* test, Fig. 2a–b). When compared to the baseline responses, the mean C-fiber-evoked field potentials 1–4 h after BDNF application were 186.9 ± 15.7% in BDNF + TAT-scramble treated rats and 102 ± 1.8% in BDNF + TAT-Fyn39-57 treated rats ($p < 0.001$, vs. BDNF + TAT-scramble group, n = 5 rats per group) (two-tailed unpaired *t* test, Fig. 2c). Similarly, the decreased PWT induced by spinal BDNF was significantly restored by the pre-treatment of TAT-Fyn39-57 (5.83 ± 0.91 g, n = 10) as compared with its control peptide TAT-scramble (13.46 ± 0.87 g, n = 11) ($p < 0.001$, two-tailed unpaired *t* test, Fig. 2d). As assessed by inclined-plate test, no significant motor dysfunction was found in rats received any above drugs (data not shown).

Taken together, these results indicate that Fyn kinase-mediated phosphorylation of GluN2B subunit at Tyr1472 is involved in BDNF-induced spinal LTP and pain hypersensitivity in intact rats.

Co-Localization of Fyn and GluN2B-Containing NMDA Receptors in Dorsal Horn Neurons of the Spinal Cord

To provide further evidence clarifying the role for Fyn kinase in the activation of GluN2B-NMDA receptors in dorsal horn neurons of the spinal cord, we examined the distribution of Fyn and GluN2B-NMDA receptor in cultured dorsal horn neurons using immunofluorescent staining. The results revealed that the expression of both Fyn and GluN2B, as well as Fyn and beta-tubulin isotype III, a widely used neuronal marker, were co-expressed in cultured dorsal horn neurons (Fig. 3), indicating a co-localization of Fyn and GluN2B-NMDA receptor in dorsal horn neurons of the spinal cord. These data presented morphological evidence to show that Fyn is a possible intermediate kinase linking BDNF/TrkB signaling with GluN2B-NMDA receptors in dorsal horn neurons of the spinal cord, and subsequently mediating their activation.

Fyn Kinase-Mediated Phosphorylation of GluN2B Subunit at Tyr1472 is Essential for Nerve Injury-Induced Mechanical Allodynia in Neuropathic Rats

To determine the role for Fyn kinase-mediated phosphorylation of GluN2B subunit at Tyr1472 in neuropathic pain development, we first examined expression of Fyn, $pFyn^{Y420}$ and $pGluN2B^{Y1472}$ in the spinal cord dorsal horn in rats subjected to SNL surgery. As shown in Fig. 4a–c, all expressions of Fyn, $pFyn^{Y420}$ as well as $pGluN2B^{Y1472}$ were statistically increased in the spinal cord dorsal horn in rats following SNL surgery. The relative intensity of Fyn and $pFyn^{Y420}$ immunoreactivity was respectively increased from day 3 (1.22 ± 0.05 SNL vs. 0.99 ± 0.03 sham, $p < 0.05$) to day 21 (1.34 ± 0.06 SNL vs. 1.01 ± 0.07 sham, $p < 0.01$), and from day 7 (1.21 ± 0.09 SNL vs. 0.93 ± 0.04 sham, $p < 0.05$) to day 21 (1.25 ± 0.07 SNL vs. 0.92 ± 0.06 sham,

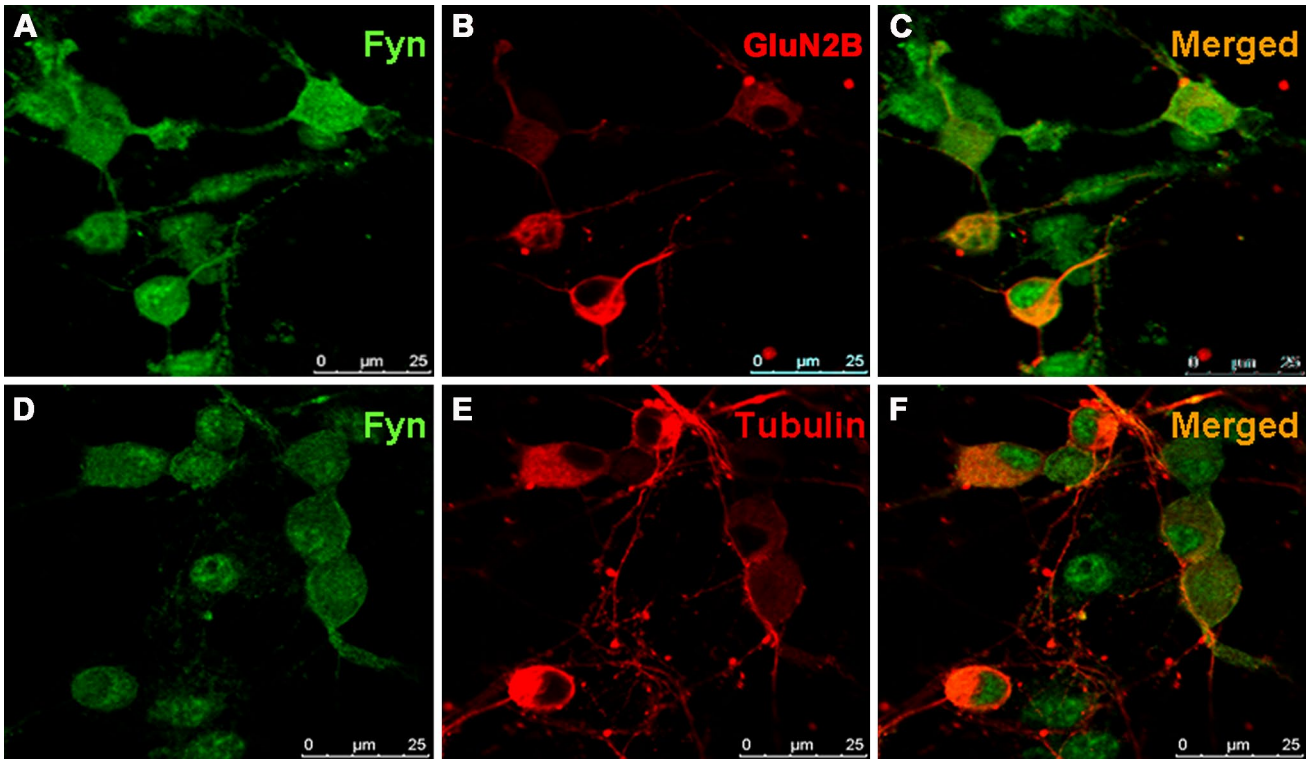
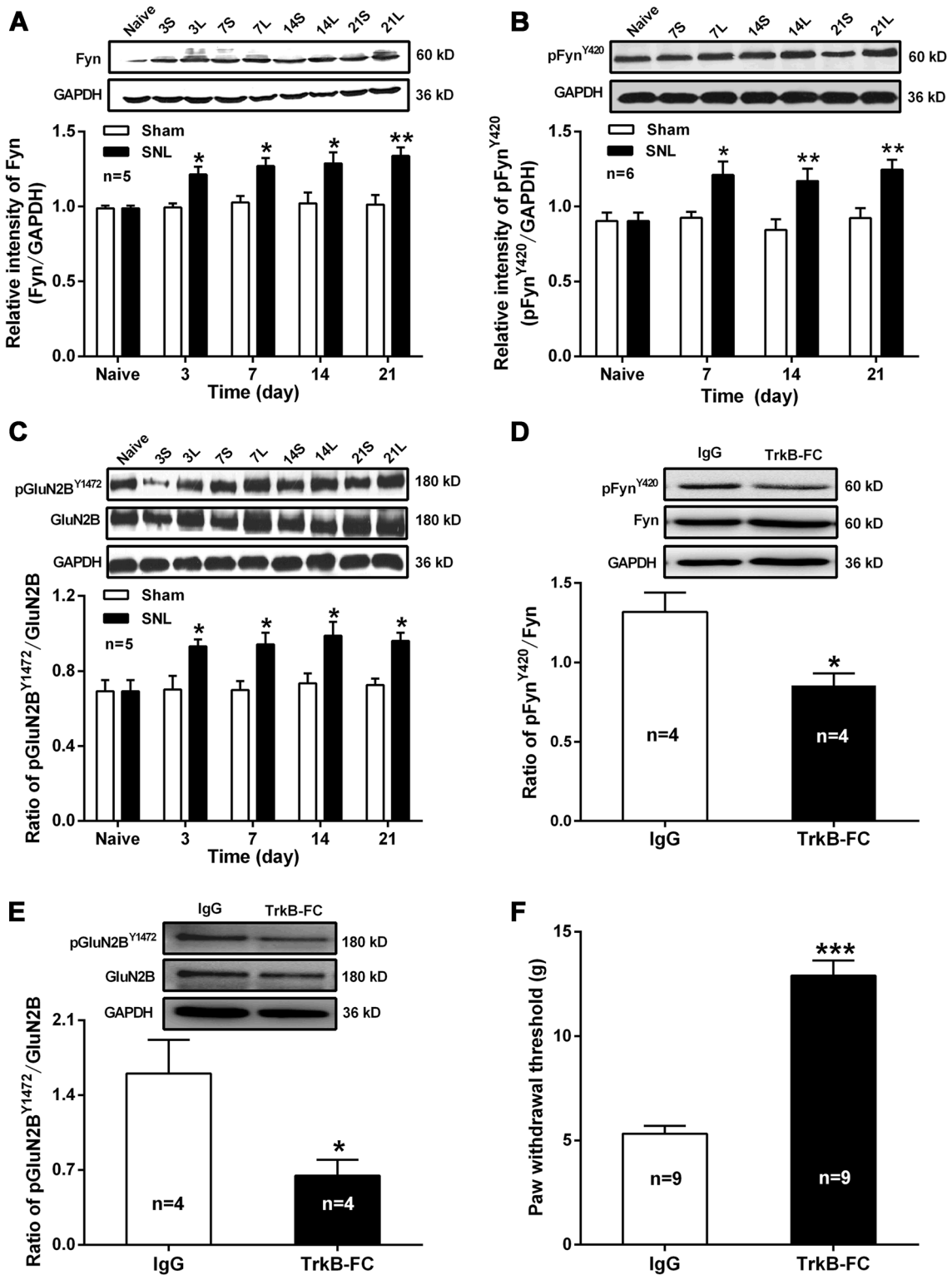


Fig. 3 Immunofluorescence staining with Fyn, GluN2B or beta-tubulin isotype III in cultured dorsal horn neurons of the spinal cord. **a**–**c** Immunostaining with anti-Fyn antibody (**a**, *green*) and anti-GluN2B antibody (**b**, *red*), respectively. Double-labeling (**c**) was created by merging the images obtained for Fyn immunostaining (*green*) and GluN2B immunostaining (*red*). **d**–**f** Immunostaining with anti-Fyn antibody (**d**, *green*) and anti-beta-tubulin isotype III antibody (**e**, *red*), respectively. Double-labeling (**f**) was created by merging the images obtained for Fyn immunostaining (*green*) and tubulin immunostaining (*red*). Note that a co-localization of Fyn with GluN2B as well as with beta-tubulin, a widely used neuronal marker, are seen in cultured dorsal horn neurons. *Scale bar* 25 μm. (Color figure online)

$p < 0.01$) post-surgery in SNL rats compared with sham rats (two-way ANOVA, $n = 5$–6 per group, Fig. 4a–b). Similarly, the ratio of $pGluN2B^{Y1472}$/GluN2B also was elevated from day 3 (0.93 ± 0.04 SNL vs. 0.70 ± 0.07 sham, $p < 0.05$) to day 21 (0.96 ± 0.04 SNL vs. 0.73 ± 0.03 sham, $p < 0.05$) post-surgery in SNL rats compared with sham rats (two-way ANOVA, $n = 5$ per group, Fig. 4c).

To further demonstrate whether BDNF is involved in the increase of $pFyn^{Y420}$ and $pGluN2B^{Y1472}$ in rats subjected to SNL surgery, we examined effects of pre-treatment with TrkB-FC on expression of $pFyn^{Y420}$, $pGluN2B^{Y1472}$ as well as on the pain hypersensitivity in SNL rats. The results showed that intrathecal injection of TrkB-FC prior to SNL surgery could significantly prevent the nerve injury-induced increase of both $pFyn^{Y420}$ and $pGluN2B^{Y1472}$ expression in the spinal dorsal horn. The ratios of $pFyn^{Y420}$ to Fyn as well as $pGluN2B^{Y1472}$ to GluN2B were respectively reduced by $32.3 \pm 11.5\%$ ($p < 0.05$) and $53.1 \pm 13.9\%$ ($p < 0.05$) in TrkB-FC-treated rats (vs. IgG, two-tailed unpaired *t* test, $n = 4$ per group, Fig. 4d–e). Consistently, pre-treatment with TrkB-FC also reversed the pain allodynia in SNL rats. The ipsilateral PWT in SNL rats was increased from 5.32 ± 0.38 g of IgG-treated rats to 12.9 ± 0.72 g of TrkB-FC-treated rats ($p < 0.001$, two-tailed unpaired *t* test, $n = 9$ per group, Fig. 4f).

Moreover, we explored the role for Fyn kinase in the activation of dorsal horn $pGluN2B^{Y1472}$ in SNL rats. Using Western blot assay, we found that the relative intensity of $pGluN2B^{Y1472}$immunoreactivity in the spinal dorsal horn was statistically increased on day 14 post-surgery in SNL rats compared to sham rats (1.02 ± 0.05 SNL vs. 0.76 ± 0.07 sham, $p < 0.05$), and this increase was significantly inhibited by the pre-treatment with PP2 (1.5 μg/μl, 20 μl, as described in Methods "Measurement of drug effects" subsection) compared with its inactive analogue PP3 (0.73 ± 0.07 SNL + PP2 vs. 0.99 ± 0.04 SNL + PP3, $p < 0.05$) (one-way ANOVA, $n = 5$ per group, Fig. 5a). In addition, we also investigated whether active Fyn kinase could elevate dorsal horn $pGluN2B^{Y1472}$ in intact rats. Recombinant active Fyn (0.1 μg/μl, 20 μl) was intrathecally delivered to rats twice per day at a 12 h-interval, continued for 2 days, and Western blot assay was performed on day 3 after behavioral assessment as described in *Methods* "Measurement of drug effects" subsection. The results



revealed that the expression of dorsal horn pGluN2B^{Y1472} was dramatically increased in active Fyn-treated rats compared with vehicle-treated rats (0.98 ± 0.06 active Fyn vs. 0.67 ± 0.05 vehicle, $p < 0.01$); and also, this kind of increase was significantly inhibited by the pre-treatment with PP2 (1.5 μg/μl, 20 μl, as described in Methods "Measurement of drug effects" subsection) compared with its inactive analogue PP3 (0.67 ± 0.05 Fyn + PP2 vs. 0.94 ± 0.07 Fyn + PP3, $p < 0.05$) (one-way ANOVA, n = 5 per group, Fig. 4b).

◂**Fig. 4** Increased expression of Fyn, phosphorylated Fyn at tyrosine 420 ($pFyn^{Y420}$) and phosphorylated GluN2B at tyrosine 1472 ($pGluN2B^{Y1472}$) in rats subjected to SNL surgery, and the inhibitory effects of pre-treatment with TrkB-FC on these alterations as well as on the pain hypersensitivity in SNL rats. **a**–**b**, Western blot of Fyn (**a**), $pFyn^{Y420}$ (**b**) and $pGluN2B^{Y1472}$ (**c**) expression in ipsilateral L4 and L5 spinal dorsal horn in SNL rats. *Upper* representative of Western blot bands; *lower* analysis of the relative intensity of Fyn, $pFyn^{Y420}$, and the ratio of $pGluN2B^{Y1472}$ to GluN2B. GAPDH is used as an internal control. Note that the expression of Fyn, $pFyn^{Y420}$ and the ratio of $pGluN2B^{Y1472}$ to GluN2B are significantly increased in rats following SNL surgery. *$p < 0.05$, **$p < 0.01$, compared with sham surgery, one-way ANOVA, n = 5–6 per group. **d**–**f** Effects of pre-treatment with TrkB-FC on $pFyn^{Y420}$ (**d**) and $pGluN2B^{Y1472}$ (**e**) expression as well as on pain hypersensitivity (**f**) in SNL rats. Note that intrathecal TrkB-FC can effectively inhibit the increased ratios of $pFyn^{Y420}$ to Fyn and $pGluN2B^{Y1472}$ to GluN2B in the spinal dorsal horn, and prevent the decreased paw withdrawal threshold in SNL rats. *$p < 0.05$, ***$p < 0.001$, versus IgG, two-tailed unpaired *t* test, n = 4 per group for Western blotting and 9 per group for behavioral testing

Finally, we performed two behavioral experiments to investigate whether inhibit the activity of Fyn kinase or GluN2B-NMDA receptors could prevent neuropathic development in SNL rats, and inhibit active Fyn-induced pain hypersensitivity in intact rats. As shown in Fig. 5c, in SNL rats, pre-treatment with either PP2 (1.5 μg/μl, 20 μl) or ifenprodil (1.0 μg/μl, 20 μl) could effectively prevent the nerve injury-induced mechanical allodynia, as assessed by measuring ipsilateral PWT. For example, in line with previous findings, the ipsilateral PWT (in gram) was statistically decreased in SNL rats compared with sham rats (5.91 ± 0.34 SNL vs. 14.62 ± 0.21 sham) ($p < 0.001$, one-way ANOVA, n = 11 per group). The decreased PWT was significantly restored by the pre-treatment with PP2 (12.34 ± 0.79) or ifenprodil (13.01 ± 0.59) as compared with their respective control of PP3 (7.39 ± 0.44) and DMSO (6.08 ± 0.93) ($p < 0.001$, one-way ANOVA, n = 9–11 per group). Likewise, in intact rats, the results showed that intrathecal administration of recombinant active Fyn (0.1 μg/μl, 20 μl) could produce robust pain hypersensitivity as assessed by a decreased PWT (in gram, 7.43 ± 0.40 active Fyn vs. 14.10 ± 0.34 vehicle) ($p < 0.001$, one-way ANOVA, n = 11 per group); and also, this active Fyn kinase-induced decrease in PWT was significantly inhibited by the pre-treatment with PP2 (11.95 ± 0.68) or ifenprodil (12.43 ± 0.57) as compared with their respective control of PP3 (8.37 ± 0.61) and DMSO (7.69 ± 0.63) ($p < 0.001$, one-way ANOVA, n = 9–10 per group, Fig. 5d). Similarly, the results of inclined-plate test confirmed that intrathecal application of any above drugs had no obvious motor dysfunction to rats (data not shown).

To ensure that all the drug effects used in this study are indeed acting through BDNF-dependent mechanisms, we also examined effects of pre-treatment with antagonist like TrkB-Fc, PP2 or ifenprodil, or with the interfering peptide TAT-Fyn39-57, on baseline C-fiber-evoked field potentials and PWT in intact rats. Expectantly, no significant effect was observed either on baseline C-fiber-evoked field potentials or on paw withdrawal threshold after pre-treatment with TrkB-Fc, PP2, ifenprodil, or TAT-Fyn39-57 as compared with their respect control IgG, PP3, DMSO, or TAT-scramble ($p > 0.05$, two-tailed unpaired t-test for LTP, n = 5 per group; two-way ANOVA for PWT, n = 6–7 per group) (Supplementary Figure S1). These data indicated that the BDNF-Fyn-GluN2B signaling may be activated mainly under neuropathic condition like peripheral nerve injury, but not at normal state. We hence suggested that there is probably not a basal level of BDNF-Fyn-GluN2B signaling before administration of exogenous agents like recombinant BDNF in intact rats.

Together these data with aforementioned findings, we suggest that Fyn kinase-mediated phosphorylation of GluN2B subunit at Tyr1472 is essential for nerve injury-induced mechanical allodynia in neuropathic rats.

Discussion

We here provide multiple lines of evidence demonstrating that BDNF contributes to spinal LTP and mechanical hypersensitivity via Fyn-mediated phosphorylation of NMDA receptor GluN2B subunit at tyrosine 1472 ($pGluN2B^{Y1472}$) in rats following spinal nerve ligation. First, we documented that Fyn kinase-mediated $pGluN2B^{Y1472}$ was involved in BDNF-induced spinal LTP and pain hypersensitivity in intact rats. It has been shown that Fyn is an intermediate kinase linking BDNF/TrkB signaling with GluN2B-NMDA receptors in the process of learning and memory [30, 31, 37, 38]. Activation of Fyn kinase-mediated $pGluN2B^{Y1472}$ through BDNF/TrkB signaling results in an enhancement of both NMDA receptor activity and synaptic transmission, thereby underlying the BDNF mechanisms in learning and memory formation [39, 58, 59]. In line with these findings, our present data revealed that intrathecal administration of BDNF indeed produced an increased expression of $pFyn^{Y420}$, $pGluN2B^{Y1472}$ as well as the ratio of GluN2B to GluN2A, but not Src and GluN2A in the spinal dorsal horn in intact rats, and that all these BDNF effects were effectively blocked by pre-treatment with either TrkB-Fc or a membrane-permeable TAT-fusion interfering peptide, TAT-Fyn 39-57, to respectively inhibit the BDNF/TrkB signaling [10, 50] and Fyn kinase activity [40, 41]. These results present strong evidence to show the role for Fyn kinase in BDNF-mediated activation of $pGluN2B^{Y1472}$ in the spinal cord dorsal horn. It is well documented that Fyn kinase is critical for synaptic plasticity and many pathophysiological processes [13–17]. In the hippocampus, Fyn

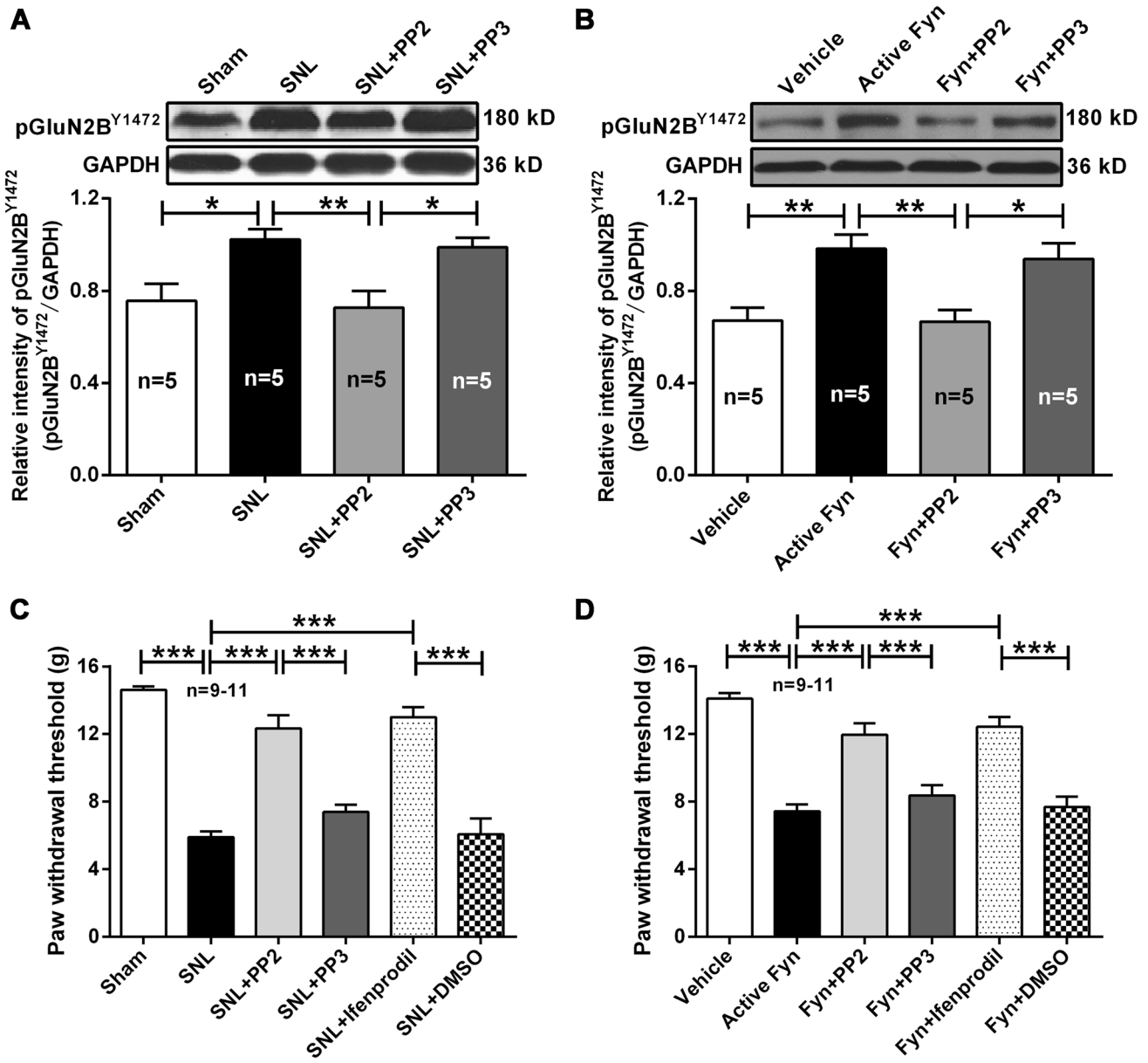


Fig. 5 Effects of pre-treatment with the SFKs inhibitor PP2 in SNL rats, or intrathecal injection of active Fyn in intact rats, on expression of pGluN2B^{Y1472} in spinal dorsal horn as well as on the painful sensitivity in rats. **a**–**b** Western blot of pGluN2B^{Y1472} expression in ipsilateral L4 and L5 spinal dorsal horn. *Upper* representative of Western blot bands; *lower* analysis of the relative intensity of pGluN2B^{Y1472}. GAPDH is used as an internal control. Note that the expression of pGluN2B^{Y1472} in spinal dorsal horn is elevated both in SNL rats and in active Fyn-treated rats, and this elevation can be significantly abrogated by pre-treatment with PP2 in rats. *p < 0.05, **p < 0.01, compared with PP3 (an inactive analogue of PP2), one-way ANOVA, n = 5 per group. **c**–**d** Effects of pre-treatment with PP2 or ifenprodil on painful sensitivity in rats subjected to SNL surgery (**c**) or received intrathecal active Fyn injection (**d**). Note that both SNL surgery and intrathecal active Fyn can produce a significant mechanical hypersensitivity as indicated by a reduction of paw withdrawal threshold (PWT) in response to von Frey filaments stimuli; and also, the decreased PWT can be reversed by pre-treatment with either PP2 or ifenprodil as compared with their respective control PP3 and DMSO. ***p < 0.001, one-way ANOVA, n = 9–11 per group

kinase-mediated pGluN2B^{Y1472} has been proved to play crucial roles in the induction of hippocampal LTP and hippocampus-dependent memory formation [19, 20]. In agreement with these findings, we currently observed that pre-treatment with either TrkB-FC, PP2, TAT-Fyn 39-57 or ifenprodil to respectively inhibit BDNF/TrkB signaling, Fyn kinase activity as well as GluN2B-NMDA receptors function, indeed abrogated the spinal BDNF-induced dorsal horn LTP and pain hypersensitivity in intact rats. These data extend previous notion to show that in the spinal cord

dorsal horn, activation of Fyn kinase also is necessary for the BDNF-induced spinal LTP and pain hypersensitivity.

The Src family of nonreceptor protein tyrosine kinases are highly expressed in neurons and are believed to play a key role in tyrosine phosphorylation of NMDA receptors [61, 62]. Fyn, a member of the Src family, has been shown to coexist with NMDA receptors at the postsynaptic density [63, 64] and directly phosphorylates NMDA subunits [65, 66], resulting in enhanced channel activity of the receptors [15, 67]. In the spinal cord, Fyn is specifically expressed in the dorsal horn, and that in the brain stem it is more characteristic in the sensory pathway, suggesting a role of Fyn in the sensory nervous network [68]. In support of this idea, we indeed found that the expression of both Fyn and GluN2B, as well as Fyn and beta-tubulin isotype III, a widely used neuronal marker, were co-existed in cultured dorsal horn neurons, indicating a co-localization of Fyn and GluN2B-NMDA receptor in the dorsal horn neurons. Here, post-synaptic density (PSD)-93, a member of the membrane-associated guanylate kinases (MAGUKs) family of proteins, has been identified to serve as a membrane-anchored substrate of Fyn and to participate in the regulation of Fyn-mediated modification of NMDA receptor function [65]. In fact, it has been revealed that PSD-93 is expressed abundantly in spinal dorsal horn and forebrain, where it colocalizes and mediates tyrosine phosphorylation of GluN2A and GluN2B through forming a complex with Fyn in the synaptosomal membrane of neurons [64]. Therefore, targeted disruption or knock-down of PSD-93 in the spinal cord results in impaired NMDAR-mediated postsynaptic functions and in blunted NMDAR-dependent persistent pain by the reduction of surface GluN2A and GluN2B expression on dorsal horn neurons [69, 70].

In the spinal cord dorsal horn, phosphorylation of GluN2B-NMDA receptors at Tyr1472 via SFKs signaling is proposed to take part in NMDA-induced pain hypersensitivity in intact mice [25]. Activation of Fyn kinase has been found to play a key role in the sustained sensitization of inflammatory pain by promoting pGluN2B^{Y1472} and recruiting GluN2B-NMDA receptors at PSD-enriched fraction in dorsal horn neurons of the spinal cord [24, 71]. In addition, Fyn kinase-mediated pGluN2B^{Y1472} also is suggested to be involved in the development of neuropathic pain in animal models of peripheral nerve injury [26–28], postherpetic neuralgia [72], or diabetic neuropathy [29]. In accordance with these findings, we indeed observed an increased expression of Fyn, pFyn^{Y420} as well as pGluN2B^{Y1472} in the spinal cord dorsal horn in rats following SNL surgery; and also, pre-treatment with the SFKs inhibitor PP2 could significantly abrogate the SNL-induced elevation of pGluN2B^{Y1472}. Moreover, our behavioral studies also revealed that in SNL rats, pre-treatment with either PP2 or ifenprodil to respectively inhibit Fyn kinase and GluN2B-NMDA receptors activity, could effectively prevent the nerve injury-induced mechanical allodynia. Our findings provide convincing evidence to support the idea that phosphorylation of GluN2B-NMDA receptors at Tyr1472 by Fyn is required for neuropathic pain development [26]. In support of this notion, we present additional evidence to show that intrathecal administration of active Fyn kinase to intact rats also could induce an increased pGluN2B^{Y1472} in spinal dorsal horn, as well as a decreased paw withdrawal threshold in response to von Frey filaments stimuli, and more important, that both actions of Fyn kinase could be blocked by the pre-treatment with either PP2 or ifenprodil.

To further determine the involvement of BDNF in the activation of Fyn kinase-mediated pGluN2B^{Y1472} and pain hypersensitivity following nerve injury, we performed additional experiments to investigate effects of pre-treatment with TrkB-FC, a TrkB-immunoglobulin G fusion protein that is often applied to scavenge endogenous BDNF [73, 74], on pFyn^{Y420} and pGluN2B^{Y1472} expression in the spinal dorsal horn, as well as on nerve injury-induced pain hypersensitivity in SNL rats. We found that intrathecal administration of TrkB-Fc prior to SNL surgery could significantly prevent the nerve injury-induced increase of both pFyn^{Y420} and pGluN2B^{Y1472} expression in the dorsal horn of the spinal cord, and also inhibit the mechanical hypersensitivity in SNL rats. These data strongly support our understanding that BDNF contributes to the activation of Fyn kinase-mediated pGluN2B^{Y1472} and pain hypersensitivity in neuropathic rats. Consistently, in a peripheral nerve injury (PNI) model of neuropathic pain, Hildebrand et al. [40] have recently demonstrated that NMDA receptor currents at lamina I synapses in the spinal dorsal horn are potentiated after nerve injury, and BDNF mediates this potentiation through phosphorylation of GluN2B by Fyn activation. In the present study, we provide additional behavioral and in vivo electrophysiological evidence to support the contribution of BDNF-Fyn-GluN2B signaling cascade to the induction of spinal LTP and pain hypersensitivity after nerve injury. Thus, disruption of this signaling pathway by pre-treatment with TrkB-FC, PP2 or ifenprodil can effectively attenuate the SNL-induced pain hypersensitivity in neuropathic rats. Our present data in fact reinforces the Hildebrand study by investigating the impacts of the BDNF-Fyn-GluN2B mechanism on spinal LTP and by testing how targeting this spinal mechanism can reverse nerve injury-induced pain hypersensitivity in vivo.

In summary, our present study suggests that the Fyn kinase-mediated phosphorylation of GluN2B subunit at Tyr1472 is critical for BDNF-induced spinal LTP and pain hypersensitivity in rats following spinal nerve ligation. Therefore, the BDNF-Fyn-GluN2B signaling cascade in the spinal cord dorsal horn may constitute a key mechanism

underlying central sensitization and neuropathic pain development after peripheral nerve injury.

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Authors' Contributions S. Li conducted Western blot and behavioral test, and participated in the design of the study and drafted the article. J. Cai performed electrophysiological experiments and the statistical analysis. Z.-B. Feng, Z.-R. Jin, H.-Y. Zhao and G.-N. Yang participated in the behavioral test and performed the statistical analysis. H.-B. Jing, T.-J. Wei, L.-Y. Liu and B.-H. Liu participated in part of immunohistochemistry and Western blot experiments. Y.-J. Cui participated in part of the design of the study. G.-G. Xing conceived of the study, participated in its design and coordination, and drafted the article. All authors have read and approved the final article.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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