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Contribution of the spinal cord BDNF to the development of neuropathic pain by activation of the NR2B-containing NMDA receptors in rats with spinal nerve ligation

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ABSTRACT

The NMDA receptor and the brain-derived neurotrophic factor (BDNF) are involved in central sensitization and synaptic plasticity in the spinal cord. To determine whether the spinal cord BDNF contributes to the development and maintenance of neuropathic pain by activation of the dorsal horn NR2B-containing NMDA (NMDA-2B) receptors, this study was designed to investigate if alterations in BDNF and its TrkB receptor in the spinal dorsal horn would parallel the timeline of the development of neuropathic pain in lumbar 5 (L5) spinal nerve ligated (SNL) rats. The enzyme-linked immunosorbent assay (ELISA) showed that the BDNF concentration significantly increased during 24 h post-surgery, and the maximal enhancement lasted for 48 h. It declined as time progressed and returned to the level of pre-operation at 28 days after SNL. In parallel with the alteration of BDNF concentration in the spinal dorsal horn, the 50% paw withdrawal threshold (PWT) of the ipsilateral hind paw in SNL rats also showed a significant decrease during 24–48 h after SNL as compared with those in sham-operated rats. The correlation analysis revealed that the BDNF concentration had a negative correlation with 50% PWT in early stage (0–48 h) ($r = -0.974$, $p = 0.001$), but not late stage (3–28 days) ($r = 0.3395$, $p = 0.6605$), after SNL. Similarly, the immunohistochemical staining revealed that a significant up-regulation of BDNF expression in the spinal dorsal horn appeared as early as 12 h post-operation in SNL rats, peaked at 24–48 h, declined at 3 days and disappeared at 14 days after SNL. In contrast, an increase in NMDA-2B receptors expression in the spinal dorsal horn was delayed to 48 h after SNL. The increase reached peak at 3 days, lasted for 14 days, and returned to the control level of pre-operation at 28 days after SNL. The maximal enhancement of BDNF expression occurred in early stage (24–48 h) after nerve injury, while the peak of NMDA-2B receptors expression appeared in late stage (3–14 days) post-nerve ligation. As compared with the dynamic changes of 50% PWT in the timeline after nerve injury, the maximal enhancement of BDNF expression closely paralleled the maximal decline in the slope of 50% PWT, while the peak of NMDA-2B receptors expression corresponded with the plateau of the decreased 50% PWT. Therefore, the increased BDNF in the spinal dorsal horn was likely to be associated with the initiation of neuropathic pain in early stage (0–48 h), while the activation of NMDA-2B receptors was involved in the maintenance of persistent pain states in late stage (2–14 days) after nerve injury. Moreover, the present study also demonstrated that the BDNF/TrkB-mediated signaling pathway within the spinal cord might be involved in the induction of neuropathic pain in early stage after nerve injury, and the selective NMDA-2B receptors antagonist (Ro 25-6981) almost completely blocked the BDNF-induced mechanical allodynia in all of the tested rats. These data suggested that the BDNF/TrkB-mediated signaling pathway in the spinal cord was involved in the development of nerve injury-induced neuropathic pain through the activation of dorsal horn NMDA-2B receptors.

Introduction

Brain-derived neurotrophic factor (BDNF), which is now established as an endogenous neuromodulator in nociceptive pathways (Thompson et al., 1999; Pezet et al., 2002b; Obata and Noguchi, 2006; Merighi et al., 2008b), may be released from primary afferent terminals within the spinal cord in an activity-dependent manner (Lever et al., 2001) and regulate nociceptive transmission in the spinal

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dorsal horn (Obata and Noguchi, 2006; Merighi et al., 2008b). Compelling evidence now shows BDNF is involved in spinal plasticity and central sensitization and therefore plays a critical role in the development of persistent pain (Kerr et al., 1999; Mannion et al., 1999; Garraway et al., 2003; Marcol et al., 2007).

It has been documented 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 ipsilateral dorsal root ganglia (DRG) neurons and the superficial dorsal horn of the spinal cord following nerve injury (Zhou et al., 1999; Shen et al., 1999; Ha et al., 2001). A marked up-regulation of tropomyosin-related kinase (TrkB) receptor in the spinal cord has also been shown in nerve injured-induced hyperalgesic mice (Narita et al., 2000). Consistent with these observations, the sciatic nerve ligation-induced thermal hyperalgesia and tactile allodynia are completely suppressed by repeated intrathecal (i.t.) injection of specific antibodies to BDNF (Yajima et al., 2002) or by TrkB-Fc chimera protein to sequester endogenous BDNF (Yajima et al., 2005). In addition, a single i.t. injection of BDNF produces a long-lasting thermal hyperalgesia and tactile allodynia in normal mice, and these responses are abolished by pre-treatment with a Trk-dependent tyrosine kinase inhibitor K-252a (Yajima et al., 2005). These findings suggest that the increased release of endogenous BDNF induced by nerve injury may cause an increase in the excitability of dorsal horn neurons through the activation of TrkB receptor and produce a neuropathic pain-like state. However, it is unclear whether the increase of BDNF in the spinal dorsal horn has an association with the timeline of the development of neuropathic pain. In the current study, we firstly investigated if alterations in BDNF and TrkB receptor in the spinal dorsal horn would parallel the timeline of the development of neuropathic pain in rats with spinal nerve ligation (SNL).

We and others have previously reported that sensory information processing in the spinal dorsal horn appears to undergo significant plastic changes such as long-term potentiation (LTP) following peripheral nerve injury or inflammation (Randic et al., 1993; Sandkuhler and Liu, 1998; Woolf and Costigan, 1999; Rygh et al., 1999; Svendsen et al., 2000; Xing et al., 2007), which are believed to be the underlying mechanisms of central sensitization and persistent pain (Sandkuhler, 2000; Ji et al., 2003; Woolf, 2007). Activation of *N*-methyl-D-aspartate (NMDA) receptors in the spinal dorsal horn has been shown to be essential for the induction of spinal LTP (Liu and Sandkuhler, 1995; Svendsen et al., 1998, 1999) and for the initiation of central sensitization (Chizh and Headley, 2005; Salter, 2005; Bleakman et al., 2006; Woolf, 2007) as well as the hyperexcitability of dorsal horn neurons in nerve injury-induced neuropathic pain (Sotgiu and Biella, 2000). Recently, several lines of evidence have shown that the released BDNF within the spinal cord results in phosphorylation and potentiation of NMDA receptors on spinal cord neurons (Kerr et al., 1999; Slack and Thompson, 2002; Slack et al., 2004), and these findings provide a possible mechanism by which BDNF mediates central sensitization (Woolf and Salter, 2000; Malcangio and Lessmann, 2003; Garraway et al., 2003). BDNF enhances the phosphorylation of NR1 and NR2B subunits of NMDA receptors in hippocampal and cortical neurons (Lin et al., 1998) and increases the open probability of NMDA receptor channels (Levine et al., 1998; Levine and Kolb, 2000). Phosphorylation of NR2B by Fyn may contribute to the increase of glutamatergic synaptic transmission induced by BDNF (Alder et al., 2005; Xu et al., 2006), and tyrosine phosphorylation of the NR2B subunit is demonstrated to be essential for the induction of LTP in the hippocampus (Nakazawa et al., 2001) as well as for the maintenance of neuropathic pain (Abe et al., 2005). More recently, we have found that NR2B-containing NMDA (NMDA-2B) receptors in the spinal cord play a crucial role in the development of central sensitization and neuropathic pain via the induction of LTP in dorsal horn nociceptive synaptic transmission (Qu et al., 2009). In the present study, we further investigated whether BDNF in the dorsal horn of the spinal cord contributes to the development and maintenance of neuropathic pain by activation of the NMDA-2B receptors.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 220–250 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 conducted 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.

Spinal nerve ligation (SNL)

Ligation of the left L5 spinal nerve was performed as described by Kim and Chung (1992). Briefly, the rats were anesthetized with chloral hydrate (0.3 g/kg, i.p.) and placed in a prone position. An incision was made into the left of the spine at the L4–S2 level. The left L5 spinal nerve was carefully isolated and tightly ligated with 4–0 silk suture 5–10 mm distal to the dorsal root ganglia and then cut approximately 2 mm distal to the suture. In control animals, sham surgery with identical procedure except for ligation of L5 spinal nerves was received. At each time point of hours 0 (pre-operation), 6, 12, 18, 24, 48 as well as days 3, 7, 14, 28 (post-operation), animals were performed for behavioral testing or sacrificed for measuring the content and protein expression of BDNF or for the expression of TrkB receptor protein as well as NR2B protein in the spinal dorsal horn. At each time point, 40 animals were randomly assigned in SNL or in sham group, respectively, in which 10–12 animals were used for behavioral test and subsequently used for ELISA test of BDNF in each time point, three animals were used for immunostaining of BDNF or NR2B protein in each time point, three animals for Western blotting of trkB protein in each time point, respectively.

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 (Xing et al., 2007; Qu et al., 2009). The 50% PWT in response to a series of von Frey filaments (Stoelting, Wood Dale, IL, USA) 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 ($18 \times 8 \times 8$ 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: $50\%\ \mathrm{PWT} = 10^{(X_f + k\delta)}/10^4$, 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. At each time point of hour 0 (pre-operation), 6, 12, 18, 24, 48 as well as day 3, 7, 14, 28 (post-operation), the mechanical allodynia was assessed by measuring 50% PWT of ipsilateral hind paw in SNL and sham-operated rats, respectively.

Intrathecal catheter implantation and drug injection

Under chloral hydrate (0.3 g/kg, i.p.) anesthesia, implantation of intrathecal cannula was performed following the method of Storkson et al. (1996). 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. Seven days after recovery from surgery, drugs or vehicle were intrathecally injected via the implanted catheter in a 20-μl volume of solution followed by 10 μl of normal saline for flushing. Each injection lasted at least 5 min. After an injection, the needle remained in situ for 2 min before being withdrawn.

Measurement of drug effects

The first behavioral experiment was performed to examine whether intrathecal (i.t.) injection of BDNF produces neuropathic pain in normal rats. BDNF at the dose of 0.1 μg in a 20-μl volume of solution, or saline in an equal volume was intrathecally administrated twice in a 30 min-interval. The 50% PWT was measured just before drug injection, and repeated measures were performed at 15, 30, 60, 90 and 120 min after drug injection, respectively.

The second experiment was preformed to determine whether the mechanical allodynia induced by i.t. injection of BDNF was dependent upon the activation of NMDA-2B receptors. To completely block NMDA-2B receptors, a selective NR2B antagonist Ro 25-6981 at 300 μg was firstly i.t. injected. Fifteen minutes later, BDNF at 0.1 μg was intrathecally administrated twice in a 30 min-interval. As a control, the equal volume of vehicle was i.t. injected at 15 min before BDNF administration. The 50% PWT was then measured and the mechanical allodynia was assessed as same as in the first experiment.

Immunohistochemical staining of BDNF, NR2B in the spinal dorsal horn

Deeply anesthetized rats were intracardially perfused with 50 ml of 0.1 M phosphate buffer (PB, PH 7.4) followed by 500 ml of Zamboni's fixative (2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0). The L4-5 lumbar segments of the spinal cord were carefully removed, post-fixed in the same fixative for 2 h and then was cryoprotected in 30% sucrose (in 0.1 M PB). Several days later, the tissues were sectioned in 40-μm thickness on a cryostat. After blocking in 10% normal goat serum for 1 h, the sections were incubated with affinity-purified rabbit antibody to recombinant human BDNF (1:1000, Chemicon, USA), or rabbit anti-NR2B antibody (1:1000, Upstate, USA) at 4 °C for 72 h. Control sections were processed without the addition of primary antibody. The sections were then washed in PBS containing 0.1% Tween 20 (PBS-T) and incubated with biotinylated goat anti-rabbit IgG (1:300, Invitrogen, USA) at 37 °C for 30 min. After several washes in PBS-T, the sections were incubated in the avidin–biotin–peroxidase complex (1:300, ABC Elite, Invitrogen, USA) at 37 °C for 45 min. The horseradish peroxidase reaction was developed in 0.1 M Tris-buffered saline (pH 7.4) containing 0.05% 3, 3P-diaminobenzidine (DAB), 0.3% nickel sulfate, and 0.01% H_2O_2. The sections were then dehydrated, mounted onto gelatin/chrome alum-coated glass slides, and coverslipped.

For relative quantification of immunoreactivity, a computer-assisted image analyzer (Image Pro Plus, version 6.0, Media Cybernetics, USA) was used at a magnification of ×20. Images were acquired using a cooled CCD camera (Spot 2; Diagnostic Instruments, Sterling Heights, MI), mounted on a Leica DMI3000 B microscope (Leica, Germany). The integrated optical density (IOD) of the immunoreactive intensity in the superficial laminae (laminae I and II) of the spinal dorsal horn was measured, and the ratio of the IOD of the ipsilateral side/IOD of the contralateral side (ipsilateral/contralateral IOD, I/C IOD) was then calculated. Four consecutive sections in 200-μm interval per L5 spinal segment were measured for each rat, and the twelve values of three rats at each time point were pooled together.

Western blot analysis of TrkB expression in the spinal dorsal horn

Western blot was performed to measure TrkB expression at the time point of day 0, 1, 2, 3, 7, 14, and 28 post-operation in SNL rats. The lumbar spinal cord was exposed by laminectomy, and approximate 2 cm length of tissue around L4-5 segment was excised and placed into a Petri dish containing dry-ice-cold homogenizing buffer. The dorsal horn was separated from the ventral horn under a dissecting microscope and immediately frozened at -80 °C and then homogenized in 1 ml ice-cold lysis buffer containing 50 mmol/L Tris (pH 7.5), 250 mmol/L NaCl, 10 mmol/L EDTA (pH 8.0), 0.5% NP40 (Sigma, MO, USA), 10 μg/ml leupeptin (Sigma), 1 mmol/L PMSF (Sigma), 4 mmol/L NaF, at 4°C for 2 h under agitation. The extracts were centrifuged at 1000 *g* for 10 min at 4 °C, and the supernatant was retained. After protein titration using a BCA protein assay kit (Pierce, Rockford, USA), the equivalent of 40 mg total protein was added to the loading buffer (2% sodium dodecyl sulfate (SDS), 100 mM DTT, 10% glycerol, 0.25% bromophenol blue) and boiled for 5 min. Proteins were separated on a 10% acrylamide resolving gel and then transferred to a PVDF membrane using a semi-dry transfer unit (BioRad, USA). After 30-min transfer in 20% methanol transfer buffer, the membranes were blocked in 5% BSA in TBS containing 0.1% Tween-20 (TBST) and were incubated in 1:1200 rabbit anti-TrkB antibody (full-length and truncated; BD Biosciences Pharmingen; USA) for more than 48 h at 4 °C. Blots were washed in TBST and then incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:1000, Jackson, USA) for 60 min at room temperature. Protein bands were visualized using an enhanced chemiluminescence detection kit (ECL™, Santa Cruz, USA) followed by autoradiography using Hyperfilm MP (Santa Cruz, USA). The standardization ratio of TrkB to β-actin band density was used to calculate the alteration in TrkB expression. For the quantification of the TrkB expression, the mean values in the control group (day 0) were converted to 100% as a baseline definition, and then individual data, including the control group, were recalculated as percent of the mean values. All of the data in Western blotting were expressed as percent of control. All experiments were performed for six times.

ELISA detection of BDNF concentration in the spinal dorsal horn

To collect tissues for BDNF analysis, the animals were anesthetized with chloral hydrate, and euthanized with an intracardiac injection of saturated potassium chloride. The lumbar spinal cord was exposed by laminectomy, and approximate 2 cm length of tissue around L4-5 segment was excised and placed into a Petri dish containing dry-ice-cold homogenizing buffer. The dorsal horn was separated from the ventral horn under a dissecting microscope and stored at -80 °C. Tissue from all of animals was processed at the same time. The dorsal horns were weighed and homogenized in cold extraction Tris-buffered saline (pH 8.0) containing 1% NP-40, 10% glycerol, 0.5 mM sodium metavanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 1 mg/ml leupeptin. The lysates were centrifuged for 15 min at 7000 *g*, and a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Promege, USA) was used to detect

BDNF in the supernatants. This monoclonal antibody shows no cross-reactivity with other neurotrophins (NT-3, NT-4, glial cell line-derived neurotrophic factor or nerve growth factor). BDNF levels in the spinal dorsal horns were normalized to the amount of total protein. The total protein in the spinal tissues was determined at the same time as the ELISA using a BCA protein assay kit (Pierce, Rockford, USA).

Chemical preparation and application

BDNF (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in sterile 0.9% saline solution at concentration of 5.0 ng/μl and given at the dose of 0.1 μg in a 20-μl volume of solution, twice in a 30-min interval. Ro 25–6981, (R-(R*,S*)-a-(4-hydroxyphenyl)-b-methyl-4-(phenylmethyl)-1-piperidine propanol (Tocris Cookson, Saint Louis, MO, USA) was dissolved in sterile 5% dimethyl sulfoxide (DMSO) with 95% saline solution at concentrations of 15 μg/μl, and given at the doses of 300 μg 15 min before BDNF administration in behavioral experiments.

Statistical analysis

All data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or two-way ANOVA followed by Bonferroni *post-hoc* test was used for multiple comparison. *F* values with their associated degrees of freedom (treatment, time, interaction and residual) were expressed

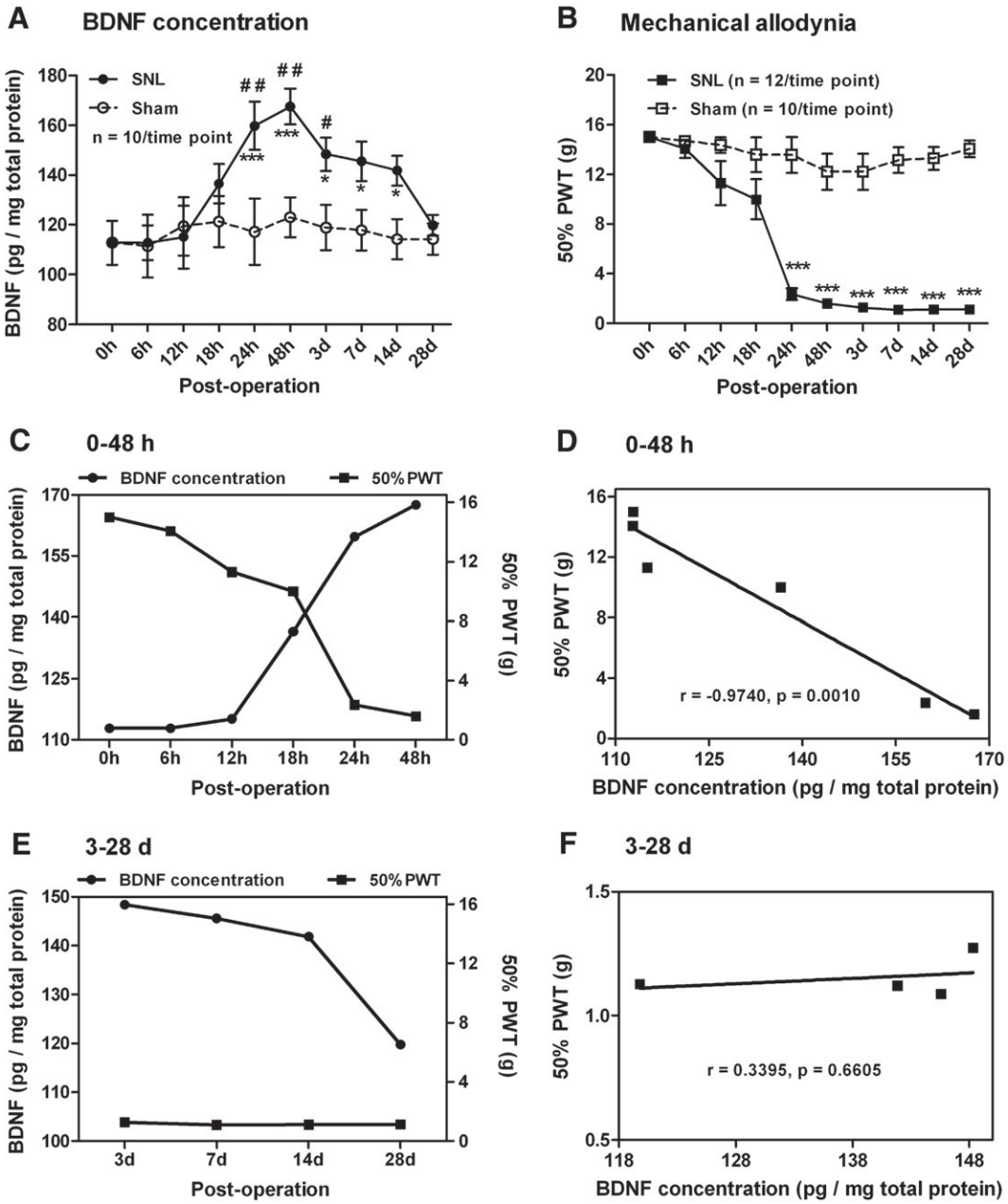

Fig. 1. Increases in the concentration of BDNF in the spinal dorsal horn were highly correlated with mechanical allodynia in early, but not late, stage after SNL. (A) Dynamic changes of the BDNF concentration (detected by ELISA) in the timeline after SNL. Note that the peak of the increased BDNF concentration occurred mainly at 24–48 h after SNL. (B) Mechanical allodynia measured by 50% PWT of the ipsilateral hind paw in SNL- and sham-operated rats. Note that the 50% PWT in SNL rats also showed a significant decrease during 24–48 h after SNL as compared with those in sham-operated rats. $^{*}p<0.05$, $^{***}p<0.001$, compared with sham-operated rats, two-way ANOVA followed by Bonferroni post-hoc test, $n=10$-12. $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with pre-operation control (0 h), one-way ANOVA followed by Dunnett's multiple comparison test, $n=10$–12/time point. (C–F) The correlation analysis between the BDNF concentration and the 50% PWT in the timeline after SNL. The BDNF concentration showed a highly negative correlation with the 50% PWT in early (0–48 h) ($r=-0.9740$, $p=0.001$, C, D), but not late (3–28 days) ($r=0.3395$, $p=0.6605$, E, F), stage after nerve injury. Data are expressed as mean ± S.E.M.

as $F_{(df \text{ of treatment, time, interaction/residual})} = F$ values (treatment, time, interaction) in two-way ANOVA, and $F_{(df \text{ of treatment, residual})} = F$ values in one-way ANOVA. Two-tailed unpaired Student's *t*-test was used for the comparison of the mean values between two groups. Correlation was analyzed between the concentration of BDNF and the 50% PWT at early (0–48 h) and late (3–28 days) stage of SNL surgery. Differences with $p<0.05$ were considered statistically significant.

Results

An increase in the concentration of BDNF in the spinal dorsal horn was highly correlated with mechanical allodynia in early, but not late, stage after nerve injury

To study whether BDNF in the spinal cord plays a role in the development of neuropathic pain, we first examined if alterations of BDNF content in the spinal dorsal horn would correlate with mechanical allodynia (as a behavioral sign of neuropathic pain) in rats with SNL. The ELISA test showed that BDNF concentration significantly increased from (112.8 ± 8.9) pg/mg total protein of pre-operation to (159.8 ± 9.7) pg/mg during 24 h post-surgery ($p<0.01$, one-way ANOVA, $F_{(9,\ 114)} = 4.34$, $n = 10$, Fig. 1A), and the maximal enhancement lasted for 48 h. The BDNF concentration declined as time progressed and returned to the level of pre-operation at 28 days after SNL. In contrast, no significant change in BDNF concentration was observed in sham-operated rats. The maximal differences in BDNF levels between the two groups of SNL- and sham-operated rats also exhibited during 24–48 h after surgery (167.6 ± 7.2 vs. 123.1 ± 7.9 pg/mg total protein at the 48-h time point, $p<0.001$, two-way ANOVA, $F_{(9,\ 1,\ 9/105)} = (1.54, 38.56, 4.21)$, $n = 10$). The difference decreased gradually from 3 days post-operation, and no significant difference was found at 28 days after surgery.

In parallel with the alteration of BDNF concentration in the spinal dorsal horn, the 50% PWT of the ipsilateral hind paw in SNL rats also showed a significant decrease during 24–48 h after SNL as compared with those in sham-operated rats (1.6 ± 0.3 vs. 12.2 ± 1.5 g at the 48 h time-point, $p<0.001$, two-way ANOVA, $F_{(1,\ 9,\ 9/200)} = (345.6, 25.95, 15.77)$, $n = 10$–12, Fig. 1B). In contrast to BDNF, the decrease in 50% PWT lasted for more than 28 days after nerve ligation.

The correlation analysis revealed that the BDNF concentration had a negative correlation with 50% PWT in early (0–48 h) ($r = -0.9740$, $p = 0.0010$, Figs. 1C, D), but not late (3–28 days) ($r = 0.3395$, $p = 0.6605$, Figs. 1E, F) stage after SNL. These results suggested that the increased BDNF in the spinal dorsal horn was correlated with initiation, but not maintenance, of neuropathic pain after spinal nerve injury.

An increase in BDNF protein expression in the spinal dorsal horn was mainly detected in early stage after nerve injury

In consistency with the alteration of BDNF concentration assessed by ELISA test, the increase in BDNF protein expression in the spinal

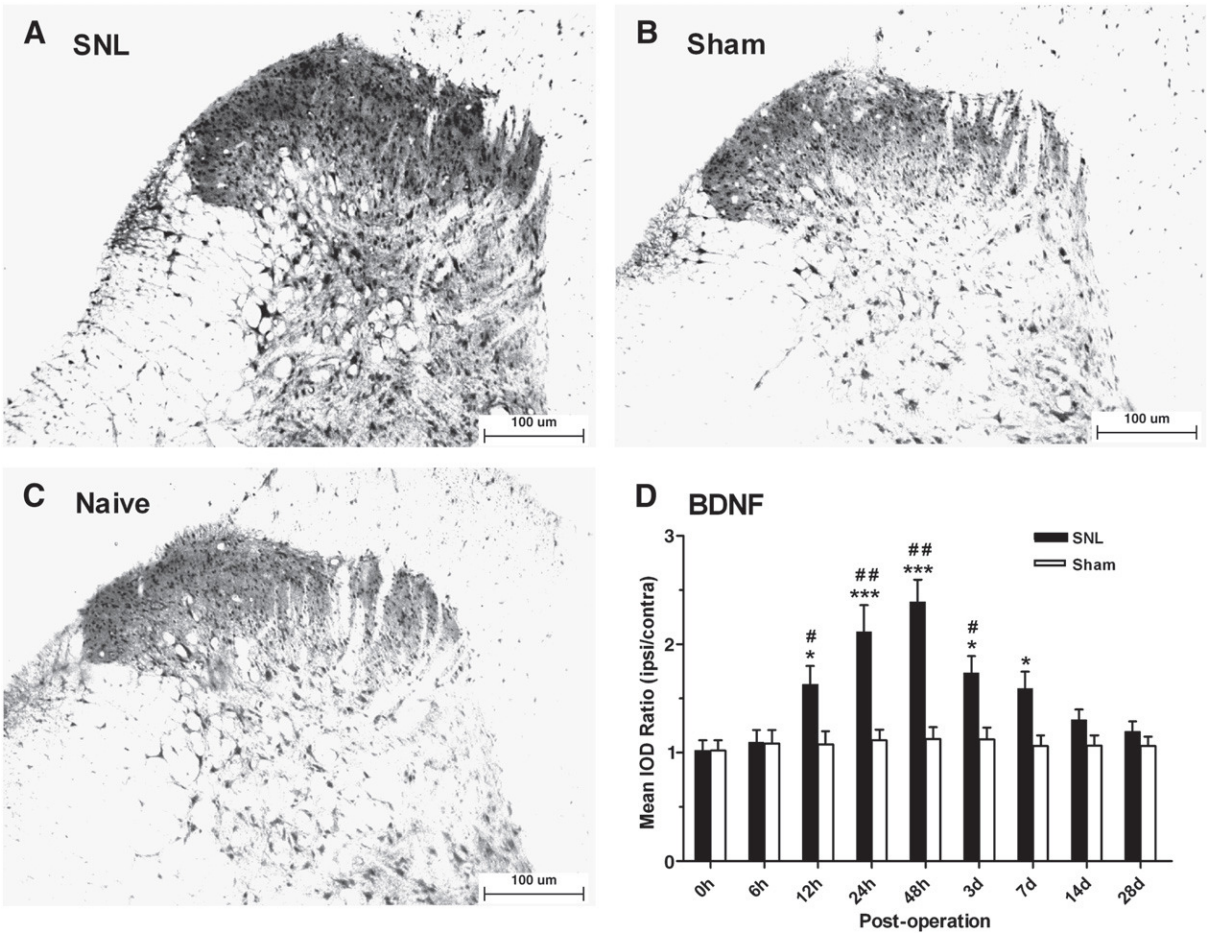

Fig. 2. Dynamic changes of BDNF protein expression in the spinal dorsal horn in the timeline post-nerve ligation. Immunohistochemical staining showed that SNL caused a significant up-regulation of BDNF protein expression in the superficial laminae (laminae I and II) of the spinal dorsal horn in SNL (A), but neither in sham-operated (B) nor in naïve rats (C). Note that the maximal increase of BDNF protein expression in the spinal dorsal horn was also observed in early stage (24–48 h) after SNL (D). $^{*}p<0.05$, $^{***}p<0.001$, compared with sham-operated rats, two-way ANOVA followed by Bonferroni post-hoc test, $n = 12$. $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with pre-operation control (0 h), one-way ANOVA followed by Dunnett's multiple comparison test, $n = 12$. Data are expressed as mean ± SEM. Scale bar = 100 μm.

dorsal horn was also observed in early stage after nerve ligation. As shown in Fig. 2, a significant up-regulation of BDNF protein expression in the spinal dorsal horn appeared as early as 12 h post-operation in SNL, but neither in sham-operated nor in naïve rats. As compared with pre-operation control, the mean integrated optical density (IOD) (ipsilateral/contralateral IOD, I/C IOD) ratio of the immunoreactive intensity in the superficial laminae (laminae I and II) of the spinal dorsal horn increased from 1.0 ± 0.1 of pre-operation to 1.6 ± 0.2 at 12 h ($p<0.05$) and peaked to 2.4 ± 0.2 at 48 h ($p<0.01$) after SNL. The enhancement declined to 1.7 ± 0.2 at 3 days ($p<0.05$) and disappeared at 7 days (1.6 ± 0.2, $p>0.05$) after nerve injury (one-way ANOVA, $F_{(8,\ 99)} = 9.1$, $n = 12$, Fig. 2D). Similar dynamic changes were also found as compared with sham-operated rats. Significant increase in the mean I/C IOD ratio appeared at 12 h (1.6 ± 0.2 vs. 1.1 ± 0.1, $p<0.05$), peaked at 24–48 h (2.4 ± 0.2 vs. 1.1 ± 0.1 at the 48 h time-point, $p<0.001$), declined at 3 days (1.7 ± 0.2 vs. 1.1 ± 0.1, $p<0.05$), and disappeared at 14 days (1.3 ± 0.1 vs. 1.1 ± 0.1, $p>0.05$) after SNL (two-way ANOVA, $F_{(1,\ 8,\ 8/198)} = (60.44, 7.03, 5.54)$, $n = 12$, Fig. 2D).

An increase in NMDA-2B receptors expression in the spinal dorsal horn was mainly detected in late stage after nerve injury

In contrast to BDNF protein expression, an increase in NMDA-2B receptor expression in the spinal dorsal horn was mainly detected in late, but not early, stage after SNL surgery. As shown in Fig. 3, significant increase in the mean I/C IOD ratio of the immunoreactive intensity in the superficial laminae of the spinal dorsal horn was delayed to 48 h after nerve ligation (1.7 ± 0.2 vs. 1.0 ± 0.1 of pre-operation, $p<0.01$). The increase reached peak at 3 days (2.4 ± 0.2, $p<0.001$), lasted for 14 days, (1.8 ± 0.2, $p<0.001$), and returned to the control level of pre-operation at 28 days (1.4 ± 0.1, $p>0.05$) after SNL (one-way ANOVA, $F_{(8,\ 99)} = 13.26$, $n = 12$, Fig. 3D). A significant difference was also found at 48 h post-operation between SNL- and sham-operated rats (1.7 ± 0.2 *vs.* 1.1 ± 0.1, $p<0.01$). The difference reached to peak at 3 days (2.4 ± 0.2 vs. 1.1 ± 0.1, $p<0.001$), lasted for 14 days (1.1 ± 0.1, $p<0.001$), and disappeared at 28 days (1.4 ± 0.1 vs. 1.1 ± 0.1, $p>0.05$) after SNL (two-way ANOVA, $F_{(1,\ 8,\ 8/198)} = (90.74, 10.90, 9.95)$, $n = 12$, Fig. 3D).

To determine whether the increased BDNF in the spinal dorsal horn plays a role in initiation, while the activation of NMDA-2B receptors in maintenance of neuropathic pain, we compared the dynamic changes between the BDNF and the NMDA-2B receptors expressions in the timeline after nerve ligation. As shown in Fig. 4A, the maximal enhancement of BDNF expression occurred in early stage (24–48 h) after nerve injury, while the peak of NMDA-2B receptors expression appeared in late stage (3–14 days) post-nerve ligation. This peak had an obvious rightward shift than that of BDNF expression in the timeline. As compared with the dynamic changes of 50% PWT in the timeline after nerve injury, the maximal enhancement of BDNF expression closely paralleled the maximal decline in the slope of 50% PWT, while the peak of NMDA-2B

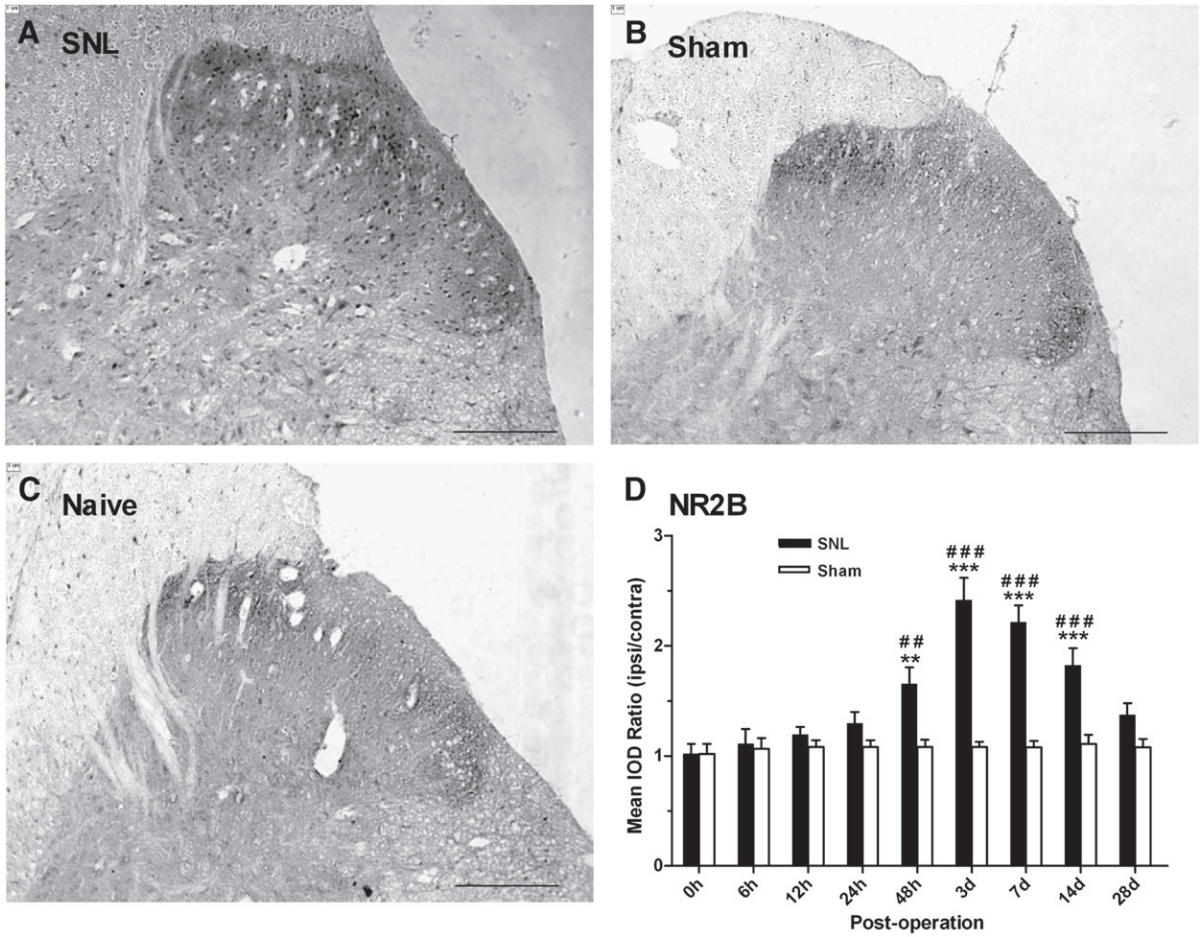

Fig. 3. Dynamic changes of NMDA-2B receptors in the spinal dorsal horn in the timeline post-nerve ligation. Immunohistochemical staining showed that SNL caused a significant up-regulation of NMDA-2B receptors expression in the superficial of the spinal dorsal horn in SNL (A), but neither in sham-operated (B) nor in naïve rats (C). Note that the maximal increase of NMDA-2B receptors expression in the spinal dorsal horn was mainly detected in late (3–14 days), but not early, (24–48 h) stage after SNL (D). $^{**}p<0.01$, $^{***}p<0.001$, compared with sham-operated rats, two-way ANOVA followed by Bonferroni post-hoc test, $n = 12$. $^{\#\#}p<0.05$, $^{\#\#\#}p<0.001$, compared with pre-operation control (0 h), one-way ANOVA followed by Dunnett's multiple comparison test, $n = 12$. Data are expressed as mean ± SEM. Scale bar = 100 μm.

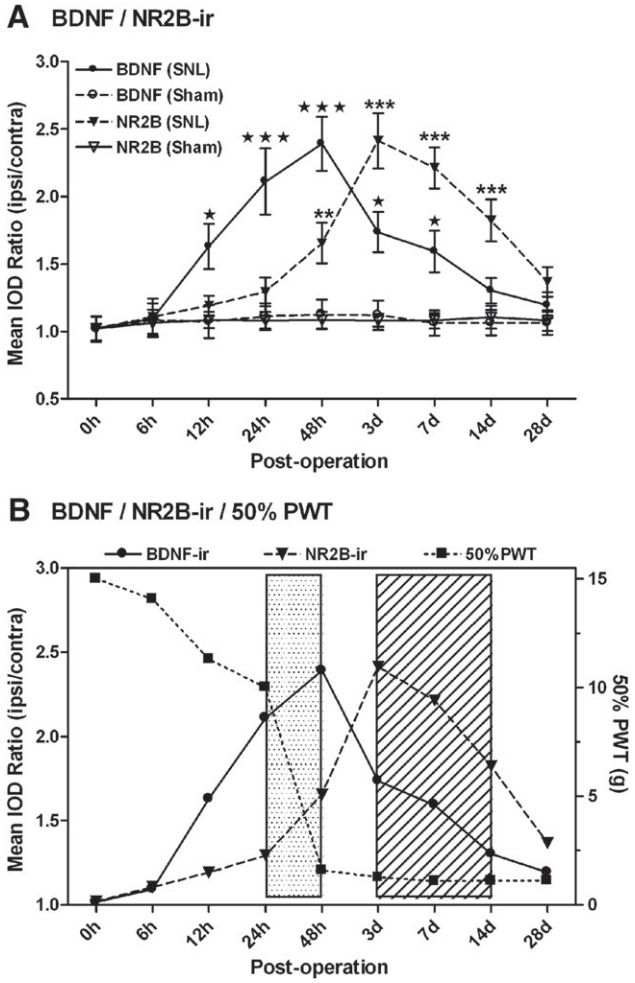

Fig. 4. Comparisons of the dynamic changes among expressions of BDNF and NMDA-2B receptors in the spinal dorsal horn and the 50% PWT in the timeline post nerve injury. (A) The dynamic changes of BDNF and NMDA-2B receptors expression in the spinal dorsal horn. Note that the maximal enhancement of BDNF expression occurred in early stage (24–48 h), while the peak of the NMDA-2B receptors expression appeared in late stage (3–14 days) after SNL. This peak had an obvious rightward shift than that of BDNF expression in the timeline. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with sham-operated rats, two-way ANOVA followed by Bonferroni post-hoc test, $n=12$. (B) As compared with the dynamic changes of 50% PWT in the timeline after SNL, the peak of BDNF expression closely paralleled the maximal decline in the slope of 50% PWT, while the maximal enhancement of the NMDA-2B receptors expression corresponded with the plateau of the decreased 50% PWT. Data are expressed as mean ± SEM.

receptors expression corresponded with the plateau of the decreased 50% PWT (Fig. 4B). These results indicated that the increased BDNF in the spinal dorsal horn was likely to be associated with the induction of neuropathic pain in early stage, while activation of NMDA-2B receptors was involved in the maintenance of persistent pain states in late stage after nerve injury.

Activation of TrkB receptors in the spinal dorsal horn in rats with nerve injury-induced neuropathic pain

To investigate whether the BDNF/TrkB-mediated signaling pathway within the spinal cord was involved in the development of neuropathic pain, we also detected the changes of TrkB receptors expression in the spinal dorsal horn in the timeline after nerve ligation. A significant increase in the expression of full-length TrkB (FL.TrkB) was observed at day 1 (208.7 ± 11.5% of control, $p<0.001$), and reached peak at day 2 (237.1 ± 12.1% of control, $p<0.001$) after SNL. The immunoreactivity of the FL.TrkB was then decreased at day 3 (163.5 ± 13.9% of control, $p<0.01$), and returned to the control level of pre-operation at day 14 (124.5 ± 10.1% of control, $p>0.05$) after nerve injury (one-way ANOVA, $F_{(6,\ 65)}=20.79$, $n=6$, Fig. 5). These changes were similar to those in BDNF concentration and protein expression in the timeline after nerve ligation, suggesting that the BDNF/TrkB-mediated signaling pathway within the spinal cord was likely to be involved in the initiation of neuropathic pain in early stage after nerve injury.

The induction of BDNF-mediated mechanical allodynia was blocked by Ro 25-6981, a selective antagonist of NMDA-2B receptors

To further assess whether the increased BDNF in the spinal dorsal horn contributes to the development of nerve injury-induced neuropathic pain through activation of NMDA-2B receptors, we first examined the effects of spinal administration of BDNF on 50% PWT (a sign for the assessment of mechanical allodynia) in normal rats. The results showed that i.t. administration of BDNF produced a significant mechanical allodynia as measured by 50% PWT in all of the tested rats. As shown in Fig. 6A, the 50% PWT decreased significantly from 14.8 ± 0.3 g of pre-administration to 9.4 ± 0.8 g at 30 min after BDNF application ($p<0.001$), and the effect lasted at least for 120 min of our experimental observation (7.1 ± 0.6 g, $p<0.001$) (one-way ANOVA, $F_{(5,\ 66)}=18.86$, $n=12$). No significant change was observed in 50% PWT in naïve rats, or after saline application. These results indicated that the spinal cord BDNF was involved in the induction of mechanical allodynia. We further determined whether the spinal BDNF-induced mechanical allodynia was dependent upon the activation of NMDA-2B receptors in the spinal dorsal horn. As our expectation, the selective NMDA-2B receptors antagonist (Ro 25-6981) almost completely blocked the BDNF-induced mechanical allodynia in all of the tested rats ($p<0.001$ compared with vehicle + BDNF) (two-way ANOVA, $F_{(2,\ 5,\ 10186)}=(247.6,\ 23.32,\ 20.97)$, $n=11$–12, Fig. 6B). These data provided direct evidence that the spinal cord BDNF was involved in the development of neuropathic pain through the activation of dorsal horn NMDA-2B receptors.

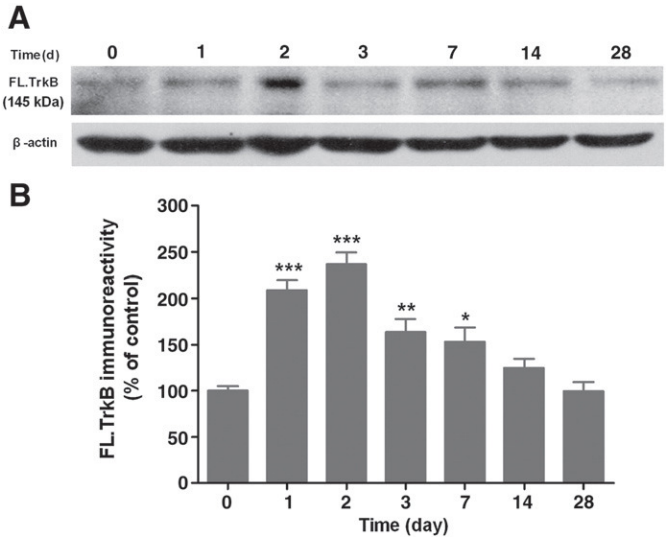

Fig. 5. Western blotting detection of TrkB expression in the spinal dorsal horn in the timeline post nerve injury. (A) Western blotting bands. Upper row: full-length TrkB (FL. TrkB); Lower row: β-actin. (B) Analysis of optical band density. Note that SNL caused a significant increase in the expression of FL.TrkB at 1 day ($p<0.001$), and reached peak at 2 days ($p<0.001$) after SNL. The immunoreactivity of the FL.TrkB was then decreased at 3 days ($p<0.01$) and returned to the control level of pre-operation at 14 days ($p>0.05$) after SNL. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with pre-operation control (0 h), one-way ANOVA followed by Dunnett's multiple comparison test, $n=6$. Data are expressed as mean ± SEM.

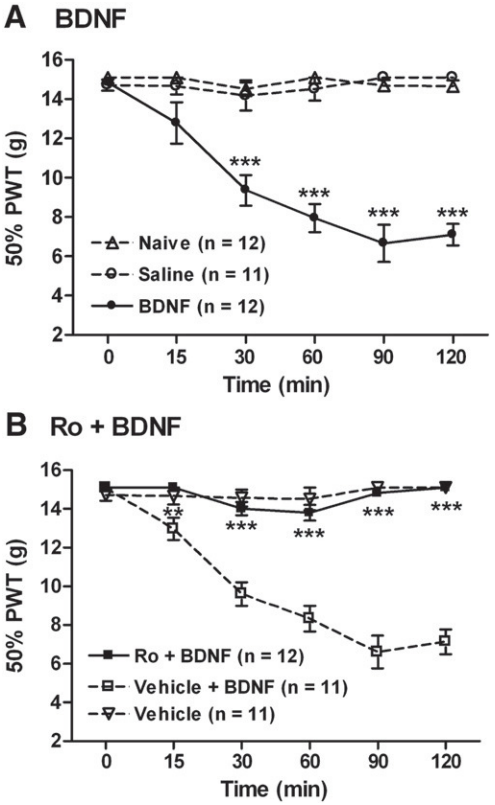

Fig. 6. The induction of BDNF-mediated mechanical allodynia was blocked by Ro 25-6981, a selective antagonist of NMDA-2B receptors. (A) Effects of i.t. injection of BDNF (0.1 μg, twice in a 30-min interval) on 50% PWT in normal rats. Note that i.t. BDNF produced a significant mechanical allodynia as measured by 50% PWT in all of the tested rats. $^{***}p<0.001$, compared with pre-operation control (0 h), one-way ANOVA followed by Dunnett's multiple comparison test, $n=12$. (B) Effects of Ro 25-6981 on the BDNF-induced mechanical allodynia. Note that the selective antagonist of NMDA-2B receptors (Ro 25-6981) almost completely blocked the BDNF-induced mechanical allodynia in all of the tested rats. $^{**}p<0.01$, $^{***}p<0.001$, compared to vehicle + BDNF, two-way ANOVA followed by Bonferroni post-hoc test, $n=11$–12. Data are expressed as mean ± SEM.

Discussion

In the present study, we found for the first time to our knowledge that the early increased BDNF in the spinal dorsal horn was likely to be associated with the initiation of neuropathic pain, while activation of NMDA-2B receptors was involved in the maintenance of persistent pain states after nerve injury. Moreover, the present study also demonstrated that the BDNF/TrkB-mediated signaling pathway within the spinal cord might be involved in the induction of neuropathic pain in early stage after SNL, and the selective NMDA-2B receptors antagonist (Ro 25-6981) almost completely blocked the BDNF induced mechanical allodynia in all of the tested rats. These data provided direct evidence that the spinal cord BDNF/TrkB-mediated signaling pathway was involved in the development of nerve injury-induced neuropathic pain through the activation of dorsal horn NMDA-2B receptors.

Role of the spinal cord BDNF in the initiation of nerve injury-induced neuropathic pain

BDNF is a member of the neurotrophin family and plays important roles in survival, differentiation and synaptic plasticity of neurons (Merighi et al., 2008b). Considerable evidence has accumulated that BDNF is also involved in the modulation of spinal nociception (Pezet et al., 2002b; Obata and Noguchi, 2006; Pezet and McMahon, 2006). The changes of BDNF expression appear to occur in the DRG and spinal cord in some pathological pain states. For example, in various inflammatory pain models, BDNF is up-regulated in the DRG and spinal cord, and sequestering the up-regulated BDNF reduces the pain hypersensitivity (Cho et al., 1997; Kerr et al., 1999; Groth and Aanonsen, 2002; Matayoshi et al., 2005; Duric and McCarson, 2007). Similarly, in nerve injury-induced neuropathic pain models, BDNF increases in the medium- and large-sized DRG neurons and their central terminals as well as the activated microglia in the spinal cord (Cho et al., 1998; Ha et al., 2001; Coull et al., 2005; Tsuda et al., 2005; Boudes and Menigoz, 2009). Intrathecal (i.t.) injection of a TrkB-Fc chimera protein, which sequesters endogenous BDNF, or delivery of BDNF antibodies reduces the nerve injury-induced neuropathic pain behaviors (Zhou et al., 2000; Yajima et al., 2002, 2005). More recently, it has been reported that the BDNF is up-regulated in the ipsilateral lumbar DRG and spinal cord following hind paw incision in rats, and i.t. injection of BDNF antibody greatly inhibits the mechanical allodynia induced by incision (Li et al., 2008). In consistency with these observations, our current study demonstrated that both of the concentration and protein expression of BDNF dramatically increased within the spinal cord after SNL. Interestingly, the present study also found that the increased BDNF mainly appeared in early stage after nerve injury. The correlation analysis revealed that the increased BDNF within the spinal cord was correlated with mechanical allodynia only in early, but not late, stage in rats with SNL. These results suggested that the increased BDNF in the spinal dorsal horn was probably correlated with initiation, but not maintenance, of neuropathic pain. Moreover, previous study has found the increases in BDNF concentration in the spinal dorsal horn parallel the timeline of the development and disappearance of thermal hyperalgesia in rats with loose ligation of the sciatic nerve (Miletic and Miletic, 2002). The data provide further support to the notion that BDNF may significantly contribute to injury-induced plasticity in sensory information processing in the spinal dorsal horn, and ultimately to the development of persistent pain (Obata and Noguchi, 2006; Merighi et al., 2008b).

The BDNF/TrkB signaling pathway is likely to be involved in synaptic mechanisms underlying both memory and pain (Yamada and Nabeshima, 2003; Malcangio and Lessmann, 2003; Bramham and Messaoudi, 2005; Merighi et al., 2008a; Wang et al., 2009). Supporting of this notion, agonists of TrkB receptor with local administration into the plantar surface induce thermal hyperalgesia in rats (Shu et al., 1999). Peripheral nerve injury induced by the partial ligation of the sciatic nerve produce a marked hyperalgesia as well as a clearly up-regulation of TrkB receptor in mice (Narita et al., 2000). Noxious chemical, mechanical, or thermal stimuli, but not innocuous stimuli, cause TrkB phosphorylation in the spinal dorsal horn (Pezet et al., 2002a). Exogenous BDNF induces a rapid activation of ERK, and sequestering BDNF with a TrkB-IgG fusion molecule significantly reduces the activation of ERK evoked by noxious stimulation (Pezet et al., 2002a; Lever et al., 2003). These data suggest that BDNF exerts a neuromodulatory role in pain processing through stimulation of postsynaptic TrkB receptors and subsequent activation of downstream signaling pathways (Obata and Noguchi, 2006; Merighi et al., 2008b). In the present study, we further confirmed that a significant increase in the expression of full-length TrkB (FL.TrkB) in the spinal dorsal horn occurred from day 1 to day 7 and peaked within the first 2 days after nerve injury. These changes matched well with the alterations of BDNF concentration and protein expression in the timeline after nerve ligation, suggesting that the BDNF/TrkB-mediated signaling pathway within the spinal cord was likely to be involved in the initiation of neuropathic pain.

Contribution of the spinal cord BDNF to the development of neuropathic pain by activation of the NMDA-2B receptors

It is well established that BDNF exerts its effects via interactions with other receptors and ion channels (Schinder and Poo, 2000; Chao,

2003; Rose et al., 2004; Ren and Dubner, 2007). For example, the BDNF-mediated potentiation of excitatory transmission in the spinal dorsal horn appears to be associated with the activation of post-synaptic NMDA receptors (Garraway et al., 2003; Merighi et al., 2008b). 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; Slack and Thompson, 2002; Slack et al., 2004; Obata and Noguchi, 2006), and this effect represents a possible mechanism by which BDNF mediates central sensitization (Malcangio and Lessmann, 2003; Garraway et al., 2003; Bramham and Messaoudi, 2005; Zhou et al., 2008; Wang et al., 2009). Previously, we have found that the spinal cord NMDA-2B receptors play a crucial role in the development of central sensitization and neuropathic pain via the induction of LTP in dorsal horn nociceptive synaptic transmission (Qu et al., 2009). Several lines of evidence also indicate that the NMDA-2B receptor is likely to be one of the targets of BDNF-induced modulation of synaptic plasticity (Lin et al., 1998, 1999; Crozier et al., 1999; Levine and Kolb, 2000; Di et al., 2001; Kim et al., 2006; Caldeira et al., 2007b). For instance, BDNF rapidly and transiently enhances tyrosine phosphorylation of the NMDA receptor subunits NR1 and NR2B in isolated cortical and hippocampal postsynaptic densities (PSDs) (Suen et al., 1997; Lin et al., 1998, 1999) as well as purified PSDs from rat spinal cord (Di et al., 2001), which result in an enhancement of NMDA receptor activity (Levine and Kolb, 2000; Xu et al., 2006; Kim et al., 2006; Caldeira et al., 2007b). Moreover, phosphorylation of NR2B by Fyn is suggested to contribute to the increase of glutamatergic synaptic transmission by BDNF (Alder et al., 2005), and tyrosine phosphorylation of the NR2B subunit is also associated with LTP in the hippocampal CA1 region (Nakazawa et al., 2001, 2002). Of particular relevance, blockade of the NR2B subunit prevents the BDNF-induced enhancement of glutamatergic neurotransmission (Crozier et al., 1999). Thus, the NMDA-2B receptor is particularly crucial to the effects of BDNF on the long-term synaptic plasticity as well as learning and memory (Mizuno et al., 2003; Malcangio and Lessmann, 2003; Alder et al., 2005; Bramham and Messaoudi, 2005) and ultimately to the development of BDNF-mediated central sensitization and persistent pain (Garraway et al., 2003; Yajima et al., 2005; Obata and Noguchi, 2006; Lu et al., 2007; Zhou et al., 2008; Merighi et al., 2008b). In support of this notion, the present study further found that the maximal enhancement of BDNF expression occurred in early stage (24–48 h) after SNL, while the peak of the NMDA-2B receptors expression appeared in late stage (3–14 days) after nerve injury. This peak had an obvious rightward shift than that of the BDNF expression in the timeline. Compared to the dynamic changes of 50% PWT in the timeline after nerve injury, the peak of the BDNF expression closely paralleled the maximal decline slope of the 50% PWT, while the peak of the NMDA-2B receptors expression corresponded with the plateau of the decreased 50% PWT. In addition, our present study also found that the increases in the concentration of BDNF in the spinal dorsal horn were correlated with mechanical allodynia only in early (0–48 h), but not late (3–28 days), stage in rats with SNL surgery. Moreover, our current data demonstrated that the selective NMDA-2B receptors antagonist (Ro 25-6981) almost completely blocked the BDNF-induced mechanical allodynia in all of the tested rats, indicating that BDNF contributed to the induction of neuropathic pain by activation of NMDA-2B receptors in the spinal dorsal horn. However, the NR2B protein was significantly high compared to sham only from day 2 to day 14. Obviously at day 28, BDNF and NR2B were normal, but mechanical hyperalgesia was still retained maximum. We speculate that early upregulation of BDNF is linked to the onset of mechanical hyperalgesia, and increased expression of NR2B correlates to its maintenance during 2–14 days after nerve injury. Other mechanisms, like protein synthesis and AMPA receptors (AMPAR) trafficking, are also likely involved in the maintenance of spinal central sensitization that underlies persistent pain after 14 days of nerve ligation (Garry and Fleetwood-Walker, 2004; Galan et al., 2004; Park et al., 2009). Emerging evidence suggests that AMPAR subunit trafficking is critically involved in activity-induced late-LTP (Yao et al., 2008; Vlachos et al., 2008) and synaptic plasticity (Liu and Zukin, 2007; Santos et al., 2009). Indeed, the GluR1 AMPAR subunit membrane insertion and GluR2 internalization in dorsal horn neurons participate in the maintenance of NMDA receptor-dependent nociceptive hypersensitivity in persistent inflammatory pain (Park et al., 2008, 2009). Activation of synaptic NMDA receptors induces membrane insertion of new GluR1 AMPAR (Lu et al., 2001; Pickard et al., 2001) or internalization of GluR2 AMPAR (Tigaret et al., 2006) in cultured hippocampal neurons, while BDNF activates AMPAR trafficking into synaptic sites (Itami et al., 2003; Caldeira et al., 2007a) or mediated GluR1 tyrosine phosphorylation potentially regulates synaptic plasticity through NMDA-2B receptors (Wu et al., 2004). The BDNF-induced synaptic delivery of GluR1 and GluR4 AMPAR is differentially mediated in NMDAR-independent or NMDAR-dependent manner, respectively (Li and Keifer, 2009). These findings suggest that the BDNF-induced AMPAR trafficking in the dorsal horn neurons might contribute to the maintenance of persistent pain in the late stage after 14 days of nerve ligation, in which phase the NMDA-2B receptors occurs down-regulation (Vikman et al., 2008).

In summary, the present study provided direct evidence that the increased BDNF in the spinal dorsal horn was likely to be associated with the induction of neuropathic pain in early stage, while the activation of the NMDA-2B receptors was involved in the maintenance of persistent pain states in late stage after nerve injury. This study suggested that the BDNF/TrkB-mediated signaling pathway in the spinal cord was likely to be involved in the initiation and development of nerve injury-induced neuropathic pain through the activation of dorsal horn NMDA-2B receptors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.expneurol.2010.01.003.

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