Brain Research Bulletin 127 (2016) 56–65

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Brain-derived neurotrophic factor (BDNF) in the rostral anterior cingulate cortex (rACC) contributes to neuropathic spontaneous pain-related aversion via NR2B receptors

Le Zhang [1], Gongming Wang [1], Jinben Ma, Chengxiao Liu, Xijiang Liu, Yufeng Zhan, Mengyuan Zhang (PhD.) *

Department of Anesthesiology, Shandong Provincal Hospital affiliated to Shandong University, Jinan, 250021, China

ARTICLE INFO

Article history:
Received 18 May 2016
Received in revised form 3 August 2016
Accepted 25 August 2016
Available online 27 August 2016

Keywords:
BDNF
rACC
NR2B
Pain-related aversion
Conditioned place preference

ABSTRACT

The rostral anterior cingulate cortex (rACC) plays an important role in pain affect. Previous investigations have reported that the rACC mediates the negative affective component of inflammatory pain and contributed to the aversive state of nerve injury-induced neuropathic pain. Brain-derived neurotrophic factor (BDNF), an activity-dependent neuromodulator in the adult brain, is believed to play a role in the development and maintenance of inflammatory and neuropathic pain in the spinal cord. However, whether and how BDNF in the rACC regulates pain-related aversion due to peripheral nerve injury is largely unknown. Behaviorally, using conditioned place preference (CPP) training in rats, which is thought to reveal spontaneous pain-related aversion, we found that CPP was acquired following spinal clonidine in rats with partial sciatic nerve transection. Importantly, BDNF was upregulated within the rACC in of rats with nerve injury and enhanced the CPP acquisition, while a local injection of a BDNF-tropomyosin receptor kinase B (TrkB) antagonist into the rACC completely blocked this process. Finally, we demonstrated that the BDNF/TrkB pathway exerted its function by activating the NR2B receptor, which is widely accepted to be a crucial factor contributing to pain affect. In conclusion, our results demonstrate that the BDNF/TrkB-mediated signaling pathway in the rACC is involved in the development of neuropathic spontaneous pain-related aversion and that this process is dependent upon activation of NR2B receptors. These findings suggest that suppression of the BDNF-related signaling pathway in the rACC may provide a novel strategy to overcome pain-related aversion.

1. Introduction

Neuropathic pain, as one of the most common types of chronic pain, always behaves in a spontaneous manner (a continuous or paroxysmal pain that is not related to an external stimulus). This non-evoked pain is difficult to measure in animals and its pathogenesis is still poorly understood. A recent study using the principle of negative reinforcement effectively produced conditioned place preference (CPP) in nerve injured rats, which intuitionally mirrored pain-related negative affection and aversive learning produced by neuropathic spontaneous pain (King et al., 2009). In clinical practice, there has been considerable evidence suggesting that patients with chronic pain suffer from much more affective disturbances than pain itself (Crombez et al., 1999; Vlaeyen and Linton, 2000). Thus, increased attention should be paid to the treatment of pain affection.

Brain-derived neurotrophic factor (BDNF) is commonly regarded to be an activity-dependent neuronal modulator in the adult brain that enhances neuronal excitability. Several lines of evidence have demonstrated that BDNF is overexpressed after nerve injury or inflammation in related regions of pain transmission, such as the spinal cord, rostral ventromedial medulla and other cortical areas (Lin et al., 2011; Geng et al., 2010; Guo et al., 2006; Thibault et al., 2014). The increased release of endogenous BDNF is necessary for plasticity changes and central sensitization and thus contributes to the development of chronic pain (Garraway

Abbreviations: BDNF, brain-derived neurotrophic factor; CPP, conditioned place preference; CTX-B, cyclotraxin-B; F-CPA, formalin-induced conditioned place avoidance; LTP, long-term potentiation; NMDAR, Nmethyl-D-aspartate receptors; PFC, prefrontal cortex; rACC, rostral anterior cingulate cortex; SNI, spare nerve injury; TrkB, tropomyosin receptor kinase B.

* Corresponding author: Department of Anesthesiology, Shandong Provincal Hospital affiliated to Shandong University, 324# Jingwu Road, Jinan, 250021, China.
E-mail address: Zhangmy717@163.com (M. Zhang).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.brainresbull.2016.08.016
0361-9230/

et al., 2003; Obata and Noguchi, 2006; Marcol et al., 2007). It is well established that chronic pain produced by BDNF signaling mainly occurs through activation of tropomyosin receptor kinase B (TrkB) receptors (Narita et al., 2000). The hyperalgesia and tactile allodynia caused by sciatic nerve injury are completely blocked by intrathecal application of BDNF inhibitor TrkB-Fc chimera protein; moreover, hyperalgesia and tactile allodynia produced by administration of exogenous BDNF in normal mice are also completely prevented by application of a TrkB receptor antagonist, K-252a (Yajima et al., 2005). Although a great deal of research has focused on the generation of persistent pain mediated by BDNF/TrkB signaling, whether and how this signaling function in neuropathic spontaneous pain-related aversion is not well known.

Nmethyl-D-aspartate (NMDA) receptors are heteromeric complexes including the essential NR1 subunit and one or more of the NR2A-D subunits (Meguro et al., 1992). Activation of NMDA receptors in the ACC were required for the synaptic plasticity and long-term potentiation (LTP), and ultimately contributed to the development of chronic pain as well as pain-associated unpleasantness (Bliss et al., 2016; Zhuo, 2008; Zhuo, 2016). Importantly, the NMDA receptor NR2B subunits participated in nociceptive transmission and pain regulatory in the CNS and played a critical role in chronic pain formation (Yang et al., 2015; Zhuo, 2009). Recently, Geng et al. found that the BDNF/TrkB-mediated signaling pathway in the spinal cord promoted the development of neuropathic pain induced by nerve injury and that this process was dependent upon the activation of dorsal horn NR2B receptors (Geng et al., 2010). Thus, we wondered whether NR2B receptors in the rACC contribute to neuropathic spontaneous pain-related negative emotions induced by BDNF signaling.

Increasing evidence indicates that the rostral anterior cingulate cortex (rACC) plays an important role during the processing of chronic pain (Gao et al., 2004; Johansen et al., 2001; Price, 2000). Animal behavioral studies have shown that the destruction of neurons originating from the rACC prevented the aversive state and negative emotional learning induced by acute noxious stimulation (Johansen and Fields, 2004), and clinical imaging examination studies showed that the rACC area could be activated by both noxious stimuli and pain-induced unpleasantness (Bornhovd et al., 2002; Tolle et al., 1999). However, the underlying mechanism of how neuropathic spontaneous pain-related aversive states arise in the rACC is still unclear. In this study, we hypothesized that the pain-related aversion in the rACC produced by peripheral nerve injuries as well as persistent pain-induced central sensitization in the spinal cord might share a common signaling pathway. Therefore, in this study, we investigate whether BDNF/TrkB signaling in the rACC drives the formation of pain-related negative emotions by activating NR2B receptors in rats following spare nerve injury (SNI) surgery.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 200–220 g used in the present study were obtained from the Experimental Animal Center of Shandong University. Rats were housed in separated cages under a 12-h light/dark cycle (lights on at 7:00 am) with free access to food and water. The room temperature was maintained at 24 ± 1 °C, and the humidity was controlled at 40–50%. Animals were given a period of seven days to adjust to their new surroundings before experimental manipulations. In accordance with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983), all experiments were approved by the Animal Care and Use Committee of Shandong University.

2.2. Surgical procedures

2.2.1. Spare nerve injury (SNI) surgery

Spare nerve injury models were established as previously described (Decosterd and Woolf, 2000). All rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (300 mg/kg). The sciatic nerve and its three terminal branches were exposed by directly incising the skin on the lateral surface of the thigh and the deeper biceps femoris muscle. SNI surgery involves an axotomy and ligation of the common peroneal and tibial nerves without touching the sural nerve. The tibial and common peroneal nerves were ligated tightly with 5-0 silk and were severed 2–4 mm from its emergence. Any contact with or stretching of the intact sural nerve was avoided. Skin and muscles were sutured in two layers, separately, and the nerves were kept entirely flattened and transparent after this surgical procedure. Sham-operated rats receiving the same surgical procedure but without sectioning of any nerves were used as a control group.

2.2.2. Intra-rACC catheter implantation and drug injection

The implantation of a catheter was performed as previously described (Xiao et al., 2012). Under intraperitoneal chloral hydrate anesthesia, rats were firmly fastened into a brain stereotactic apparatus with the lambda and bregma at horizontal level. A 30-gauge stainless steel cannula with a 33-gauge stainless steel stylet plug was bilaterally implanted 0.5 mm above the rACC injection site [2.6 mm anterior to bregma, 0.6 mm lateral from the midline, 2.5 mm beneath the surface of the skull] or the prefrontal cortex (PFC) [2.6 mm anterior to bregma, 0.6 mm lateral from the midline, 3.7 mm beneath the surface of the skull] in-line with the atlas of Paxinos and Watson (1998). The cannula was fixed with denture cement, and all surgical procedures were performed under sterile conditions. Animals were allowed to recover for one week before the next experimental procedure. Rats showing any neurological defects after the surgical procedure were removed from the experiment.

For drug injection, rats were transiently anesthetized with isoflurane, and local microinjection was performed through a 33-gauge stainless-steel injection cannula. A 1 μL Hamilton syringe with PE-10 tubing was linked to the cannula that extended 0.5 mm over the tip of the guide cannula. A volume of 1 μL per hemisphere of either drug or vehicle was injected over a 5 min period. The injection cannula was kept in place for another 5 min to minimize diffusion of the drug along the injection syringes.

2.2.3. Intrathecal catheter implantation and drug injection

Implantation of the intrathecal cannula was performed as described by Storkson et al. (1996). Briefly, a PE-10 polyethylene catheter was inserted into the epidural space between the L5 and L6 vertebrae. Correct implantation was determined by observing the behavior of dragging or paralysis of bilateral hind limbs after injection of 2% lidocaine (0.2 mL) after complete recovery from anesthesia. The internal part of the catheter was fixed with the paravertebral muscles, and the outer part of the catheter was plugged and fixed onto the skin after wound closure and sutured at the head. All surgical procedures were performed under sterile conditions. Rats showing neurological deficits within 3 days after the catheter implantation were excluded.

Spinal drug administration was performed by injection of 25 μL of saline or 10 μg of clonidine in 25 μL of saline to elicit CPP in rats with nerve injury. Each injection lasted for at least 5 min. All injections were performed in a separate room, and rats were exposed to the CPP conditions within 5 min after injection.

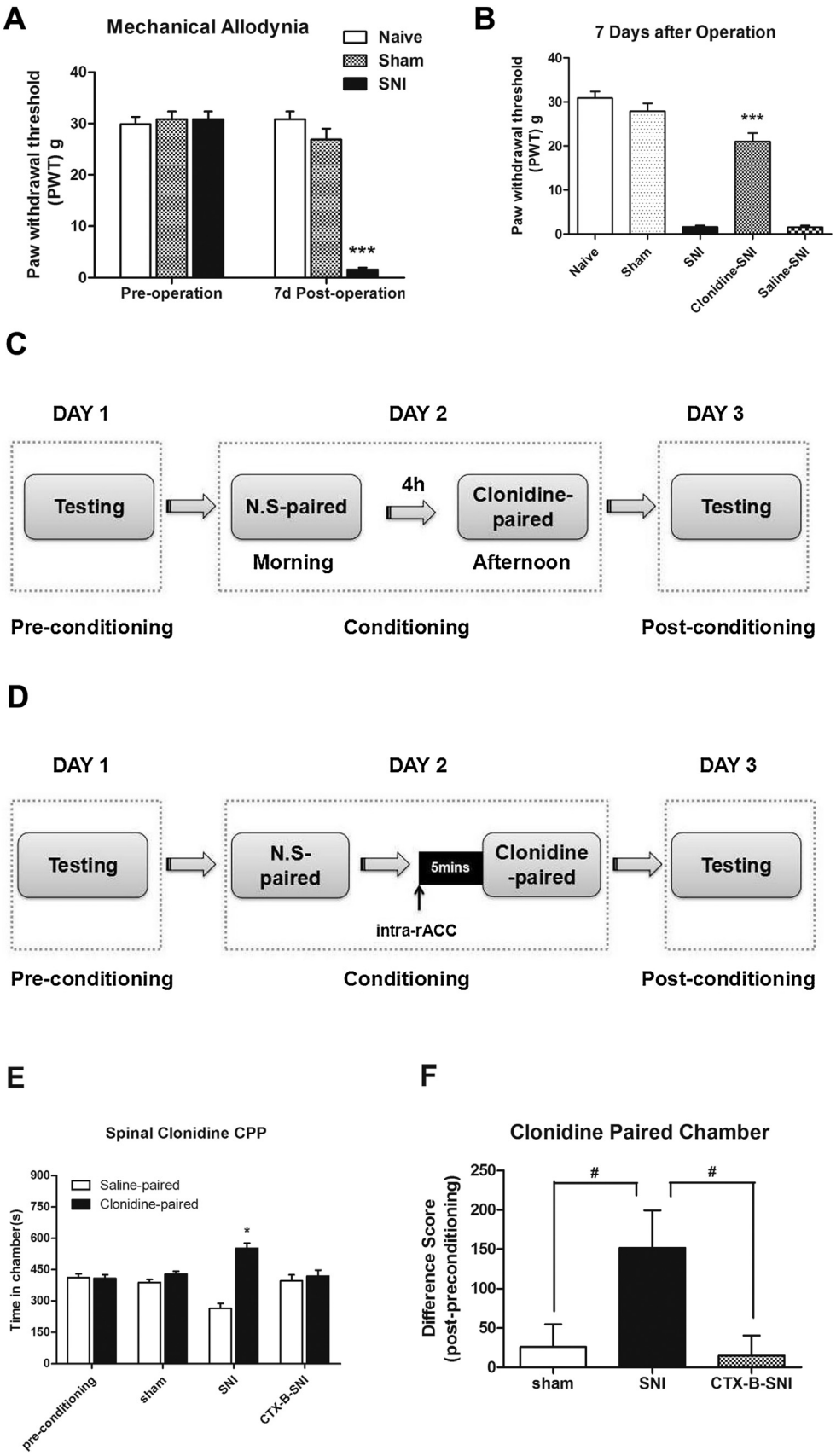

Fig. 1. (A) Mechanical allodynia measured by PWT of the ipsilateral hind paw in SNI, sham-operated and naive rats. Note that the PWT in SNI rats showed a significant decrease on day 7 after operation compared with those in control rats. ***P < 0.001, compared with pre-operation control, two-way ANOVA, $F_{(2,2,1/42)} = (59.08, 54.17, 77.66)$, n = 8/group. (B) Direct administration of clonidine into the spine significantly increased the PWT in SNI rats. ***P < 0.001, compared with the SNI rats, one-way ANOVA, $F_{(2,21)} = 90.18$, n = 8/group. Data are expressed as the mean ± SEM. (C-D) Procedure of the behavioral test (spinal clonidine CPP). (E) Surgical, sham-operated rats and SNI rats pretreated with CTX-B showed equivalent time in the pairing chambers on the pre-conditioning day. There were no differences between these groups; thus the pre-conditioning values

2.3. Behavioral studies

2.3.1. Measurement of mechanical allodynia

As previously described, mechanical allodynia was evaluated by measuring the paw withdrawal threshold (PWT) in response to a series of von-Frey filaments (Stoelting, USA) using the "up and down" method (Chaplan et al., 1994). Animals were habituated to the testing environment for 30 min. A withdrawal response was considered valid only if the hind paw was completely removed from the customized platform. The PWT was defined as the lowest hair force in grams that produced at least three withdrawal responses in five tests. Tests were performed 1 day before and on day 7 after surgery.

2.3.2. Assessment of conditioned place preference

CPP, a learned behavior that directly reflects the affective component of spontaneous pain in rats, was applied in this investigation with slight modifications. All rats were habituated to the new surroundings in the CPP conditioned box for 2 h, and during this period, they were free to access any chamber. On the first day, the movement track was recorded for 15 min and analyzed to testify whether there was a preconditioned chamber preference. Rats that spent >80% (720 s) on one side on that day were eliminated from the subsequent experiments. On the following day, rats receiving an appropriate vehicle injection were paired with a randomly chosen chamber for 30 min. Four hours after the injection, rats receiving the appropriate clonidine treatment were paired with the other chamber for 30 min. The chamber pairings were counterbalanced. On the last day, rats were placed in the CPP box with full access to any chamber, and their behavior was recorded for 15 min to analyze chamber preference and calculate the difference score (the time spent in the clonidine-paired compartment on the post-conditioning day minus that on the pre-conditioning day).

Regarding the BDNF-induced CPP (Fig. 2A), the procedures on the first day and on the last day were the same as those above mentioned. However, on the training day, exogenous BDNF (1 μg/μL, 0.5 μL per side) was administered in naive rats 5 min before CPP training, and the antagonist (CTX-B, 10 μg/μL, 0.5 μL per side; Ifenprodil, 0.2 μg/μL,0.6 μL per side) was injected into the rACC 10 min before application of BDNF. The same training was repeated for another two days.

2.4. Immunohistochemistry and immunofluorescence staining

Rats were deeply anesthetized with chloral hydrate and sacrificed by transcardial perfusion with 200 mL of 0.9% NaCl and a subsequent 200 mL of 4% paraformaldehyde (PFA), pH 7.4. Rats were completely decapitated, and brain tissues were fixed in 4% PFA at 4 °C overnight. Briefly, the rACC (AP 3.7-0.7 from Bregma) was cut into 30 μm-thick segments. On the first day, the tissue sections were treated with citric acid to retrieve antigen and were then incubated in PBS containing 10% normal goat serum and 0.3% TritonX-100 at 37 °C for 30 min. After that, sections were incubated with a primary polyclonal rabbit-anti-BDNF antibody (1:200, Abcam) at 4 °C overnight. On the second day, all sections were incubated with a biotinylated goat anti-rabbit secondary antibody at 37 °C for 30 min and then incubated with the avidin-biotin-peroxidase complex at 37 °C for 30 min. Finally, the sections were reacted with diaminobenzidine (DAB) for 1–2 min. Sections were soaked in PBS to stop the reaction, differentiated with 1% hydrochloric acid alcohol, dehydrated with 70–100% alcohol, dried in the air and cover-slipped for microscopic examination. The sections were photographed with a Leica DM4000 B microscope and a digital camera (Germany). For the relative quantification of immunoreactivity, the integrated optical density (IOD) of the immunoreactive intensity in the rACC was measured.

For TrkB/NR2B double immunofluorescence, sections were incubated with a mixture of primary rabbit anti-TrkB (1:50, Abcam) and mouse anti-NR2B (1:50, Abcam) overnight at 4 °C. On the second day, all of the steps were protected from light. Sections were incubated with a FITC-conjugated secondary antibody for 30 min at 37 °C. Then, they were incubated with a rhodamine-conjugated secondary antibody for another 30 min at 37 °C. Finally, the sections were observed and photographed in the dark room with a Leica SP2 confocal laser scanning microscope (Olympus, Japan).

2.5. Western blotting

Rats were sacrificed by overdose of chloral hydrate after CPP behavioral training, and the brain tissues were quickly removed. Briefly, brain slices containing rACC (AP 3.7-0.7 from Bregma) were cut and rapidly frozen in liquid nitrogen. Protein was exacted from frozen samples in RIPA lysis buffer containing PMSF (Sigma). Samples were centrifuged at 10000 rpm for 30 min at 4 °C after incubating in ice for 30 min. The sections were stored at −80 °C for western blotting. Equal amounts of protein (40 μg) were loaded and separated by Tris-Tricine SDS-PAGE. The resolved proteins were transferred onto polyvinilidene difluoride (PVDF) membranes. The membranes were blocked in 10% non-fat milk for 2 h at room temperature (RT) and incubated with a rabbit anti-TrkB (1:800, Abcam) or mouse anti-NR2B (1:800, Abcam) primary antibody on a shaking table overnight at 4 °C. On the next day, the blots were then incubated with a secondary antibody goat anti-rabbit IgG (1:5000, Jackson) or goat anti-mouse IgG (1:5000, Jackson) conjugated with horseradish peroxidase (HRP) for 1 h at RT. Immunoblots were visualized by using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore). Protein levels were normalized to β-actin.

2.6. Drugs

The drugs used in the present study were BDNF (PEPROTECH), Cyclotraxin (CTX-B, R&D Systems), and a selective NR2B inhibitor Ifenprodil (Selleck). BDNF and Cyclotraxin were dissolved in 0.9% sterile physiological saline. Ifenprodil was dissolved in 0.9% sterile physiological saline containing 3% dimethylsulfoxide (DMSO).

2.7. Statistical analysis

All of the data are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) or two-way ANOVA followed by the Bonferroni post-hoc test were used for multiple comparisons. A value of $P < 0.05$ was considered statistically significant. All analyses were performed using Graphpad Prism software.

3. Results

3.1. BDNF contributes to the acquisition of CPP

First, the paw withdrawal threshold (PWT) was measured on day 7 after surgery. Compared with pre-operation controls, the

were pooled for graphical representation. On the test day, only surgical rats showed a clear preference for the chamber paired with spinal clonidine (10 μg). *P < 0.05, compared with pre-conditioning, two-way ANOVA, $F_{(1,1,1/20)} = (19.71, 21.29, 10.04)$, n = 6/group. (F) Difference score, calculated as the time spent in the clonidine-paired chamber on the post-conditioning day minus that on the pre-conditioning day, indicates that only nerve injured rats increased the time spent in the clonidine-paired compartment. SNI rats pretreated with CTX-B completely blocked CPP acquisition. #P < 0.05, one-way ANOVA, $F_{(2,15)} = 4.636$, n = 6/group. Data are expressed as the mean ± SEM.

PWT was significantly decreased in surgical animals (Fig. 1A). To obtain a more definite conclusion, clonidine, a most commonly used analgesic which has been proved to be a proper way to relieve neuropathic pain, was used in this experiment. When clonidine was administered into the spine, mechanical allodynia was alleviated and the PWT was dramatically increased, which further confirmed the antinociceptive effects of clonidine (Fig. 1B).

Subsequently, spinal clonidine-induced CPP was performed (Fig. 1C-D), and the results showed that the surgical, sham-operated, and naive groups spent an equivalent preconditioning time in saline- and clonidine-paired chambers, suggesting that there was no preconditioning bias for each group (Fig. 1E). However, when clonidine (10 μg) was intrathecally administered, it caused a dramatic increase in time spent in the clonidine-paired chamber in surgical rats, but not in the sham-operated groups (Fig. 1E). The difference score (i.e., the time spent in the clonidine-paired compartment on the post-conditioning day minus that on the pre-conditioning day) between surgical and sham-operated rats was statistically significant (Fig. 1F). Additionally, the immunohistochemical results suggested that SNI dramatically increased the expression of BDNF in the rACC compared with the control animals (Fig. 2). More importantly, we also found that the bilateral microinjection of exogenous BDNF into the rACC dramatically reversed the spinal clonidine-induced pain relief in surgical animals, suggesting that the increased expression of BDNF in the rACC might play a critical role in neuropathic spontaneous pain formation (Fig. 3A).

3.2. BDNF/TrkB pathway involves in CPP development

Subsequently, to evaluate how BDNF is involved in the acquisition of CPP, we determined the expression of TrkB, the high affinity receptor of BDNF. The results showed that the TrkB protein was significantly increased in surgical rats compared with naive and sham-operated groups (Fig. 3B). To further determine whether the BDNF/TrkB mediated signaling pathway was involved in the development of affective pain, the selective TrkB receptor antagonist CTX-B (10 μg/μL, 0.5 μL per side) was bilaterally microinjected into the rACC 15 min before spinal clonidine-paired conditioning. The results indicated that CPP acquisition was mostly blocked by CTX-B in SNI rats (Fig. 1E). However, when CTX-B was bilaterally microinjected into the prefrontal cortex (PFC) instead of the rACC, CPP acquisition could not be inhibited. Together, these data indicate that BDNF within the rACC is likely to be involved in pain-related negative emotion through activation of TrkB receptors after nerve injury.

3.3. Exogenous BDNF elicits CPP by activating TrkB

To test whether BDNF is sufficient for producing CPP, exogenous BDNF (1 μg/μL, 0.5 μL per side) and an equivalent vehicle as a control were separately microinjected into the bilateral rACC of intact rats. The rats with BDNF application spent dramatically more time in the clonidine-paired context on the post-conditioning day compared with the pre-conditioning day, while the vehicle-treated rats showed no changes (Fig. 4B). However, this preference phenomenon disappeared when BDNF was bilaterally injected into the PFC area (Fig. 4D), indicating that the effect of BDNF on CPP was specifically confined to the rACC region. Furthermore, we also found that BDNF-CPP acquisition can be blocked by the pre-administration of a TrkB antagonist CTX-B (10 μg/μL, 0.5 μL per side) into the rACC (Fig. 4C).

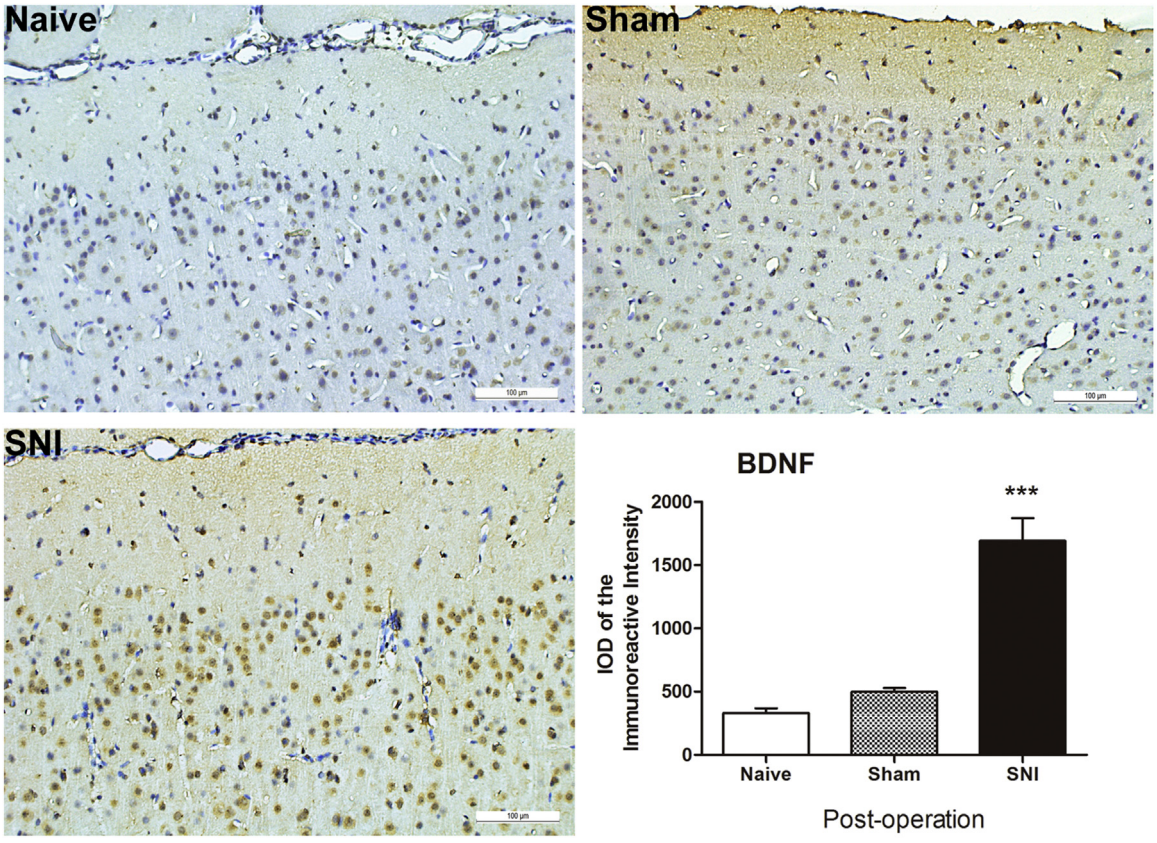

Fig. 2. Expression of BDNF protein in the rACC. Immunohistochemical staining showed that BDNF in the rACC was significantly increased in SNI rats (C), but not in sham-operated (B) nor in naive groups (A). $^{***}P < 0.001$, compared with sham-operated rats, one-way ANOVA, $F_{(2,15)} = 47.58$, Data are expressed as the mean ± SEM. Scale bar = 100 μm.

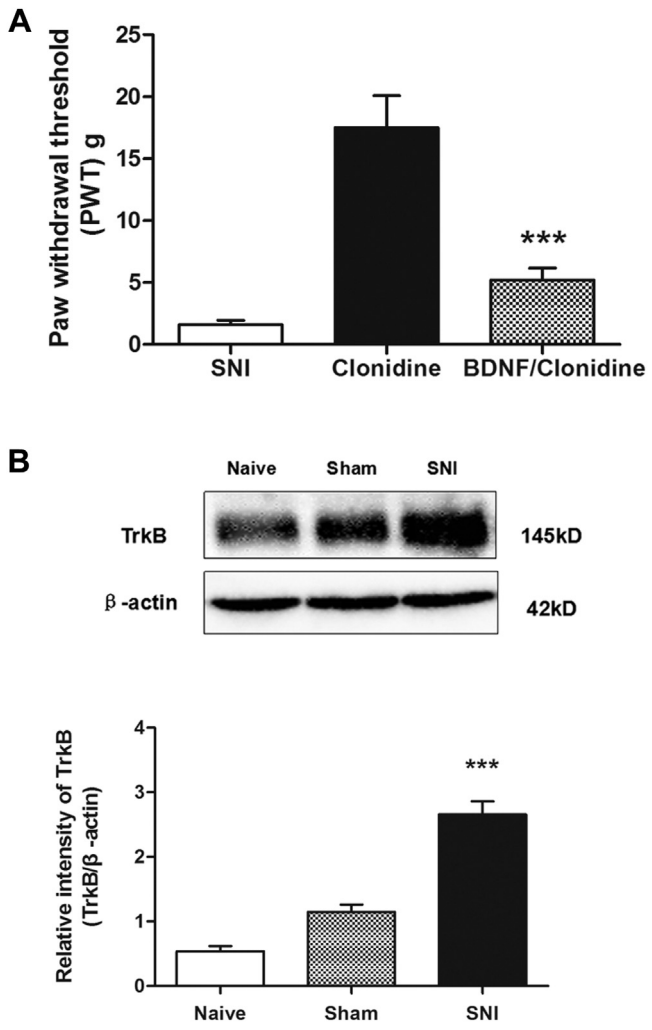

Fig. 3. (A) The spinal clonidine-induced pain relief was partially reversed by a BDNF-related compound. Intrathecal injection of clonidine dramatically increased the PWT compared with SNI rats, while bilateral microinjection of exogenous BDNF into the rACC alleviated these clonidine-induced antinociceptive effects and significantly decreased the PWT. $^{***}P < 0.001$, one-way ANOVA, $F_{(2,21)} = 26.63$, n = 8/group. Data are expressed as mean as the mean ± SEM. (B) Western blot of TrkB expression in the rACC in naive, sham-operated, SNI rats. Upper: representative of western blot bands; lower: analysis of the relative intensity of TrkB. Indicate that TrkB receptors were prominently increased in SNI rats compared with naive and sham-operated rats. $^{***}P < 0.001$, one-way ANOVA, $F_{(2,15)} = 58.76$, n = 6/group. Data are expressed as the mean ± SEM.

3.4. BDNF-related signaling upregulates NR2B expression

We further wondered whether the BDNF-induced CPP was dependent upon the activation of NR2B receptors in the rACC. Therefore, the selective NR2B receptor antagonist Ifenprodil (0.2 μg/μL, 0.6 μL per side) was locally injected into the rACC during BDNF conditioning, and the results showed that BDNF-induced CPP was completely suppressed (Fig. 4C). To provide further evidence for our hypothesis that increased BDNF in the rACC activates NR2B, which subsequently induces a pain-like aversive state, we determined the expression of NR2B receptors in the rACC. BDNF was delivered to intact rats as described in the aforementioned behavioral experiments, and then western blot analysis was carried out. As shown in Fig. 5A, we demonstrated that the NR2B expression levels were significantly increased by BDNF in the rACC following BDNF-induced CPP conditioning. The relative optical band density of NR2B immunoreactivity was prominently increased in BDNF-treated rats compared with naive and vehicle-treated groups. On the contrary, when CTX-B, the highly potent and selective TrkB inhibitor, was micro-injected into the rACC before administration of BDNF, the upregulation of NR2B receptors was completely prevented, which indicated that the NR2B expression was regulated through the BDNF/TrkB-related pathway (Fig. 5A).

3.5. BDNF/TrkB-NR2B signaling pathway in the rACC is required for the development of pain-related aversion

Subsequently, we examined whether SNI surgery would induce a functional increase of NR2B receptors through the BDNF/TrkB pathway in the rACC. As we expected, the expression of the NR2B protein was evidently increased in SNI rats compared to the naive and sham-operated groups (Fig. 5B). However, in SNI rats pretreated with CTX-B, the functional upregulation of NR2B in the rACC was mostly suppressed. The relative optical band density of NR2B immunoreactivity was significantly decreased in CTX-B pretreated rats compared with control rats (Fig. 5B). In addition, the colocalization of NR2B and TrkB receptors in rACC neurons also provided direct evidence of the involvement of NMDA receptors with the BDNF effects (Fig. 6). Taken together, these results revealed that BDNF/TrkB signaling within the rACC participated in the development of neuropathic pain via the activation of NR2B receptors.

4. Discussion

The current work is based on the assumption that the BDNF/TrkB signaling pathway in the rACC contributes to spontaneous pain-related aversion by activating NR2B receptors. Using behavioral approaches in an animal model of SNI, we found that chronic pain-related aversion produced by nerve injury is suppressed by blockage of TrkB receptors and exogenous BDNF induced CPP is completely prevented by application of NR2B receptor antagonist Ifenprodil. Finally, we validate that BDNF/TrkB-dependent neuronal transmission in the rACC is an underlying key mechanism in the negative affection of spontaneous pain due to peripheral nerve injury and that NR2B is an indispensable factor in the downstream of the signaling pathway.

Patients with neuropathic conditions usually experience ongoing and/or paroxysmal spontaneous pain (Backonja and Stacey, 2004). Spontaneous pain is a common clinical complaint, but it is difficult to find an effective measure to demonstrate pain in animals. Recently, King et al. developed a novel animal behavioral training to distinguish pain affection from pain sensation. The preference-learning task, called conditioned place preference (CPP), was induced by spinal administration of clonidine and associated with contextual cues to reflect the spontaneous pain-related aversive state (King et al., 2009). Clonidine has been proved to relieve pain in our present study, and the relief of pain is kept in memory as a rewarding signaling. Thus, the generation of CPP was based on the combination of the conditioning chamber and the rewarding memory of pain relief. The findings show that CPP was only achieved in nerve injured rats by spinal administration of clonidine and not in sham-operated or naive groups, suggesting that peripheral nerve injury caused a pain-related negative emotion. Our present study further confirmed that CPP is a valid, ideal approach to investigate spontaneous pain-related aversion without any external noxious stimuli, and we made full use of it to explore the effect of BDNF on the aversion of neuropathic spontaneous pain.

Increasing evidence has revealed that neurons in the anterior cingulate cortex (ACC) participate in emotion, learning, memory and pain-related perception (Tang et al., 2005; Maviel et al., 2004; Frankland et al., 2004), while the rACC seems to be more involved in the affective-emotional aspect of pain (Johansen et al., 2001; Johansen and Fields 2004). Previously, using conditioned place

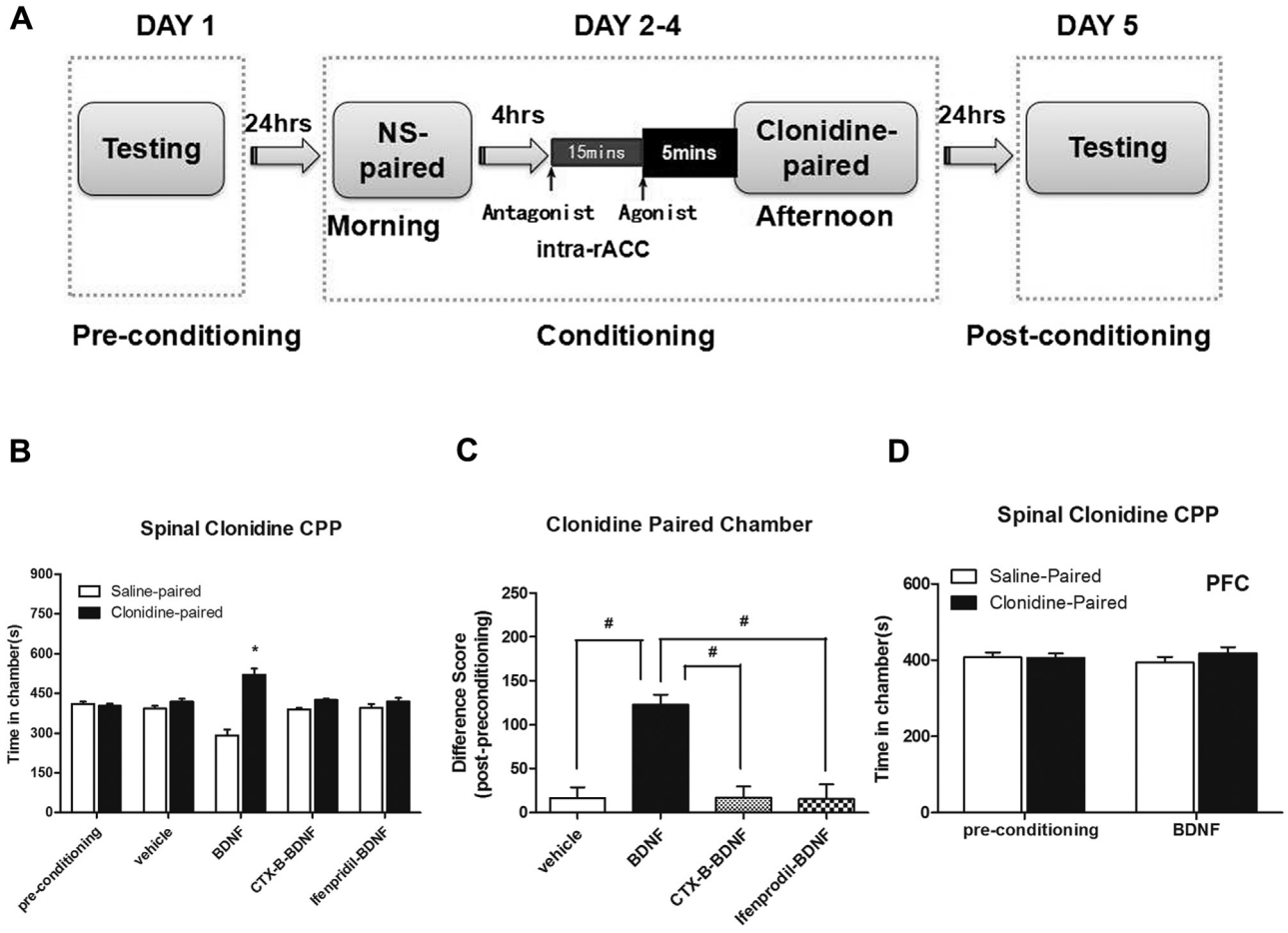

Fig. 4. Exogenous BDNF elicits CPP. (A) Schematic of the protocol for the experiments B-C. (B) Vehicle-treated rats, BDNF-treated rats, rats pre-treated with CTX-B and rats pre-treated with Ifenprodil showed equivalent time in the pairing chambers prior to the conditioning day. There were no differences in these groups; thus, the preconditioning values were pooled for graphical representation. Only BDNF-treated rats showed a clear preference for the chamber paired with spinal clonidine (10 μg). $^{*}P < 0.05$, compared with pre-conditioning, two-way ANOVA, $F_{(1,1,1/20)} = (23.25, 16.89, 10.97)$, n = 6/group. (C) Difference score, indicates that application of BDNF in intact rats increased the time spent in the clonidine-paired chamber, while pre-injection of CTX-B or Ifenprodil into the rACC attenuated the difference score. $^{\#}P < 0.05$, one-way ANOVA, $F_{(3,20)} = 15.38$, n = 6/group. Data are expressed as the mean ± SEM. (D) Intra-PFC injections of BDNF can not induce CPP in naive rats. $P > 0.05$, two-way ANOVA, $F_{(1,1,1/10)} = (0.481, 1.012, 0.362)$, n = 6/group. Data are expressed as the mean ± SEM.

preference induced by application of lidocaine in the rostral ventromedial medulla (RVM), Qu and colleagues found that the rACC mediated the affective-like responses of spontaneous chroinc pain (Qu et al., 2011). In the current study, we found that pre-treatment with the TrkB receptor antagonist CTX-B in the rACC mostly blocked CPP in SNI animals. However, when CTX-B was locally injected into the PFC, it failed to suppress the CPP. Moreover, the direct injection of BDNF into the rACC rather than PFC produced CPP, implying a critical role of the rACC in BDNF-induced aversive learning. These results therefore provide robust proof that the rACC plays a crucial role in mediating the negative emotional component of chronic pain.

BDNF is distributed across the central nervous system (CNS), including in nociceptive stimuli transmission pathways (Merighi et al., 2008; Ying et al., 2002). Currently, it is regarded as a modulator in neuropathic and inflammatory pain and involved in the initiation and development of central sensitization in the spinal cord. Geng and Leal reported that the BDNF-mediated signaling pathway in the spinal cord contributes to the induction of neuropathic pain in spinal nerve ligated (SNL) rats (Geng et al., 2010; Leal et al., 2014). TrkB, a high affinity receptor of BDNF, was also markedly upregulated in mice with peripheral nerve injury caused by a partial sciatic nerve ligation (Narita et al., 2000). Thibault et al. recently found increased BDNF expression in the ACC of rats with inflammation, while local injection of a TrkB receptor antagonist mostly alleviated neuronal hyperexcitability and prevented passive avoidance behavior, confirming that the BDNF/TrkB signaling pathway in the ACC was a tuner of the affective aspect of inflammatory pain (Thibault et al., 2014). On the basis of the above observations, we determined the expression of BDNF and TrkB and found that these two proteins dramatically increased in the rACC of SNI rats. Moreover, administration of exogenous BDNF was sufficient to induce CPP behavior, which strongly supports our hypothesis that upregulation of BDNF in the rACC potentiates the negative emotional aspect of chronic spontaneous pain. However, when the TrkB receptor antagonist CTX-B was applied, CPP acquisition was absolutely suppressed, suggesting that increased BDNF/TrkB-mediated signaling was likely to play a critical role in the development of pain-related aversion in rats that suffered a nerve injury.

The NMDA receptor is an ionotropic glutamate receptor that plays important roles in excitatory synaptic transmission. A recent study reported that an increased amount of synaptic NMDA receptors in the insular cortex contributes to the neuropathic pain formation (Qiu et al., 2013). Besides, Wu et al. demonstrated that peripheral inflammation increased the expression of NMDA receptor NR2B subunits and NR2B receptor-mediated synaptic currents in the ACC (Wu et al., 2005). Several lines of evidence have shown that BDNF is involved in pain-related affection by interacting with

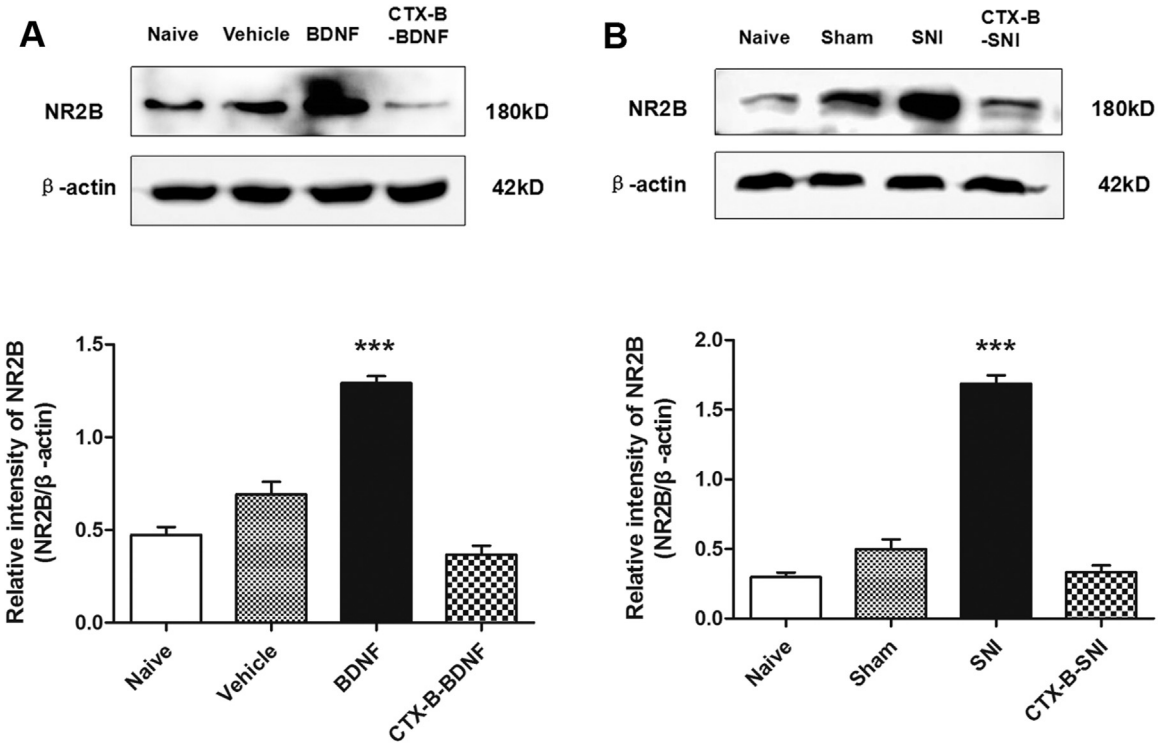

Fig. 5. Microinjection of exogenous BDNF in intact rats or SNI surgery enhance the expression of NR2B in the rACC, while pre-treatment with CTX-B prevents the upregulation of NR2B. (A) Western blot of NR2B expression in the rACC in naive, vehicle-treated, BDNF-treated rats and rats pre-treated with CTX-B. Upper: representative of western blot bands; lower: analysis of the relative intensity of NR2B. β-actin is used as internal control. Indicate that the NR2B is significantly increased in BDNF-treated rats compared with naive and vehicle-treated rats, respectively, but pre-treatment with CTX-B alleviated the increase of NR2B. $^{***}P < 0.001$, one-way ANOVA, $F_{(3,20)} = 68.42$, n = 6/group. Data are expressed as the mean ± SEM. (B) Western blot of NR2B expression in the rACC in naive, sham-operated, SNI rats, and SNI rats pre-treated with CTX-B. Upper: representative of western blot bands; lower: analysis of the relative intensity of NR2B. Note that the expression of NR2B is prominently increased in SNI rats compared with naive and sham-operated rats. Moreover, the SNI-induced up-regulation of NR2B is mostly blocked by preemptive application of CTX-B. $^{***}P < 0.001$, one-way ANOVA, $F_{(3,20)} = 139.8$, n = 6/group. Data are expressed as the mean ± SEM.

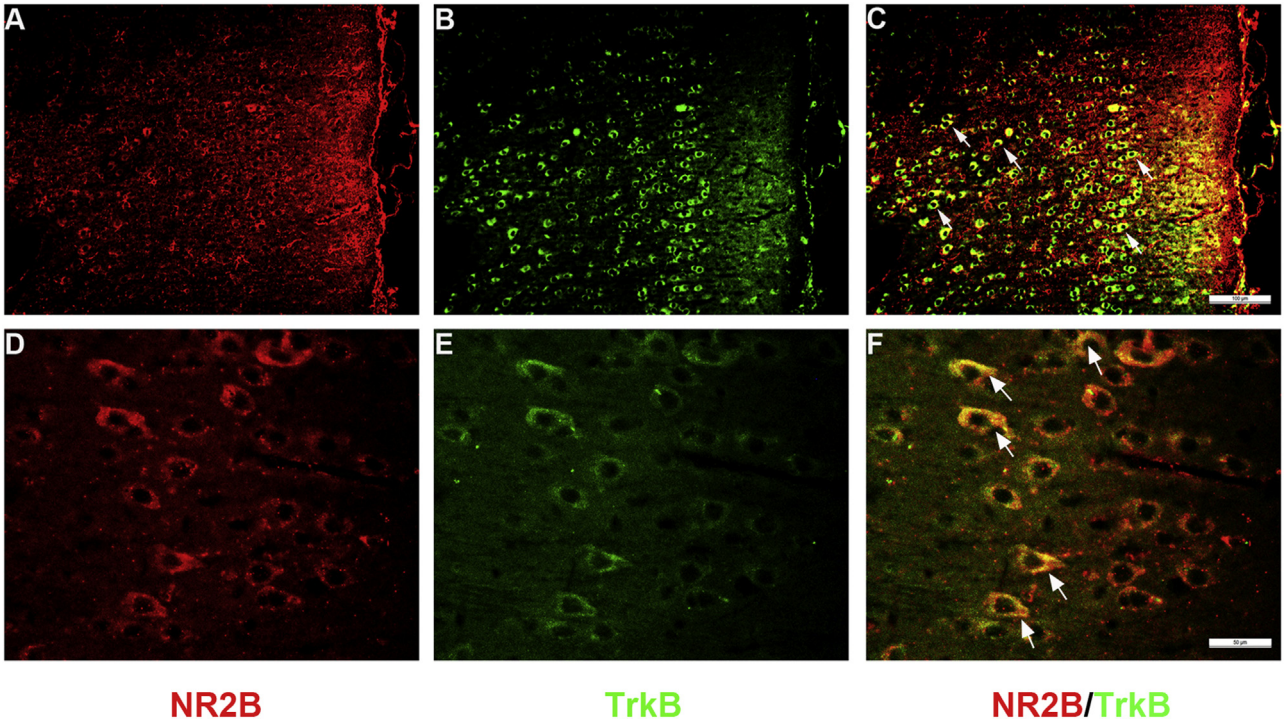

Fig. 6. Double immunofluorescence showed that NR2B (red) was co-localized with TrkB (green) receptors in the rACC under a 20x (A–C, Scale bar = 100 μm) and 40x objective (D–F, Scale bar = 50 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NR2B receptors (Caldeira et al., 2007; Kim et al., 2006). For example, BDNF was previously reported to enhance NMDA receptor activity by phosphorylating NR1 and NR2B subunits in the cortical cortex and hippocampal postsynaptic densities (Xu et al., 2006; Caldeira et al., 2007). The BDNF-induced enhancement of glutamatergic neurotransmission was prevented by blocking the NR2B subunit (Crozier et al., 1999). In addition, Ding et al. demonstrated that spinal cord BDNF took effect in central sensitization and pain hypersensitivity following nerve injury and was attributed to the activation of NR2B receptors (Ding et al., 2015). Eventually, we found that not only SNI surgery but also administration of exogenous BDNF in intact rats apparently increased the expression of NR2B receptor in the rACC, while pre-treatment with CTX-B completely alleviated the increase caused by BDNF. Moreover, pre-application of the selective NR2B receptors antagonist Ifenprodil almost completely suppressed BDNF-induced CPP. All of these results validated that the BDNF/TrkB pathway contributed to the development of neuropathic pain-related negative emotions via activation of NR2B subunits in the rACC.

To our knowledge, this is the first study to show that increased BDNF in the rACC is involved in the aversive state of neuropathic spontaneous pain and that NR2B is an important downstream effector of the BDNF/TrkB signaling pathway, which significantly contributes to this process. This research confers a clinical significance that a new strategy targeting BDNF and relative receptors in the rACC might be useful for the prevention of pain-related emotional disturbance due to peripheral nerve injury.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The present work was supported by the Natural Science Foundation of Shandong Province (2013ZRB14282), the Key Research and Development Project of Shandong Province (2014GGH218033) and the National Natural Science Foundation of China (NSFC, 30872433).

References

Backonja, M.M., Stacey, B.J., 2004. Neuropathic pain symptoms relative to overall pain rating. Pain 5, 491–497.
Bliss, T.V.P., Collingridge, G.L., Kaang, B.K., Zhuo, M., 2016. Synaptic plasticity in the anterior cingulate cortex in acute and chronic pain. Nat. Neurosci., 1–12.
Bornhovd, K., Quate, M., Glauche, V., Bromm, B., Weiller, C., Buchel, C., 2002. Painful stimuli evoke different stimulus-response function in the amygdala, prefrontal, insula and somatosensory cortex: a single-trial fMRI study. Brain 125, 1326–1336.
Caldeira, M.V., Melo, C.V., Pereira, D.B., Carvalho, R.F., Carvalho, A.L., Duarte, C.B., 2007. BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons. Mol. Cell. Neurosci. 35, 208–219.
Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53, 55–63.
Crombez, G., Vlaeyen, J.W., Heuts, P.H., Lysens, R., 1999. Pain-related fear is more disabling than pain itself: evidence on the role of painrelated fear in chronic back pain disability. Pain 80, 329–339.
Crozier, R.A., Black, I.B., Plummer, M.R., 1999. Blockade of NR2B-containing NMDA receptors prevents BDNF enhancement of glutamatergic transmission in hippocampal neurons. Learn. Mem. 6, 257–266.
Decosterd, I., Woolf, C.J., 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87, 149–158.
Ding, X., Cai, J., Li, S., Liu, X.D., Wan, Y., Xing, G.G., 2015. BDNF contributes to the development of neuropathic pain by induction of spinal long-term potentiation via SHP2 associated GluN2B-containing NMDA receptors activation in rats with spinal nerve ligation. Neurobiol. Dis. 73, 428–451.
Frankland, P.W., Bontempi, B., Talton, L.E., Kaczmarek, L., Silva, A.J., 2004. The involvement of the anterior cingulate cortex in remote contextual fear memory. Science 304, 881–883.
Gao, Y.J., Ren, W.H., Zhang, Y.Q., Zhao, Z.Q., 2004. Contributions of the anterior cingulate cortex and amygdala to pain- and fear-conditioned place avoidance in rats. Pain 110, 343–353.
Garraway, S.M., Petruska, J.C., Mendell, L.M., 2003. BDNF sensitizes the response of lamina II neurons to high threshold primary afferent inputs. Eur. J. Neurosci. 18, 2467–2476.
Geng, S.J., Liao, F.F., Dang, W.H., Ding, X., Liu, X.D., Cai, J., Han, J.S., Wan, Y., Xing, G.G., 2010. 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. Exp. Neurol. 222, 256–266.
Guo, W., Robbins, M.T., Wei, F., Zou, S., Dubner, R., Ren, K., 2006. Supraspinal brain-derived neurotrophic factor signaling: a novel mechanism for descending pain facilitation. J. Neurosci. 26, 126–137.
Johansen, J.P., Fields, H.L., 2004. Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal. Nat. Neurosci. 7, 398–403.
Johansen, J.P., Fields, H.L., Manning, B.H., 2001. The affective component of pain in rodents: direct evidence for a contribution of the anterior cingulate cortex. Proc. Natl. Acad. Sci. U. S. A. 98, 8077–8082.
Kim, Y.I., Choi, H.J., Colwell, C.S., 2006. Brain-derived neurotrophic factor regulation of Nmethyl-D-aspartate receptor-mediated synaptic currents in suprachiasmatic nucleus neurons. J. Neurosci. Res. 84, 1512–1520.
King, T., Vera-Portocarrero, L., Gutierrez, T., Vanderah, T.W., Dussor, G., Lai, J., Fields, H.L., Porreca, F., 2009. Unmasking the tonic-aversive state in neuropathic pain. Nat. Neurosci. 12, 1364–1366.
Leal, G., Comprido, D., Duarte, C.B., 2014. BDNF-induced local protein synthesis and synaptic plasticity. Neuropharmacology 76, 639–656.
Lin, Y.T., Ro, L.S., Wang, H.L., Chen, J.C., 2011. Up-regulation of dorsal root ganglia BDNF and trkB receptor in inflammatory pain: an in vivo and in vitro study. J. Neuroinflamm. 8, 126.
Marcol, W., Kotulska, K., Larysz-Brysz, M., Kowalik, J.L., 2007. BDNF contributes to animal model neuropathic pain after peripheral nerve transection. Neurosurg. Rev. 30, 235–243.
Maviel, T., Durkin, T.P., Menzaghi, F., Bontempi, B., 2004. Sites of neocortical reorganization critical for remote spatial memory. Science 305, 96–99.
Meguro, H., Mori, H., Araki, K., Kushiya, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K., Mishina, M., 1992. Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. Nature 357, 70–74.
Merighi, A., Salio, C., Ghirri, A., Lossi, L., Ferrini, F., Betelli, C., Bardoni, R., 2008. BDNF as a pain modulator. Prog. Neurobiol. 85, 297–317.
Narita, M., Yajima, Y., Aoki, T., Ozaki, S., Mizoguchi, H., Tseng, L.F., Suzuki, T., 2000. Upregulation of the TrkB receptor in mice injured by the partial ligation of the sciatic nerve. Eur. J. Pharmacol. 401, 187–190.
Obata, K., Noguchi, K., 2006. BDNF in sensory neurons and chronic pain. Neurosci. Res. 55, 1–10.
Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates. Academic Press, New York.
Price, D.D., 2000. Psychological and neural mechanisms of the affective dimension of pain. Science 288, 1769–1772.
Qiu, S., Chen, T., Koga, K., Guo, Y.Y., Xu, H., Song, Q., Wang, J.J., Descalzi, G., Kaang, B.K., Luo, J.H., Zhuo, M., Zhao, M.G., 2013. An increase in synaptic NMDA receptors in the insular cortex contributes to neuropathic pain. Sci. Signal. 6 (275), 1–11.
Qu, C.L., King, T., Okun, A., Lai, J., Fields, H.L., Porreca, F., 2011. Lesion of the rostral anterior cingulate cortex eliminates the aversiveness of spontaneous neuropathic pain following partial or complete axotomy. Pain 152, 1641–1648.
Storkson, R.V., Kjorsvik, A., Tjolsen, A., Hole, K., 1996. Lumbar catheterization of the spinal subarachnoid space in the rat. J. Neurosci. Methods 65, 167–172.
Tang, J., Ko, S., Ding, H.K., Qiu, C.S., Calejesan, A.A., Zhuo, M., 2005. Pavlovian fear memory induced by activation in the anterior cingulate cortex. Mol. Pain 1 (6), 1–16.
Thibault, K., Lin, W.K., Rancillac, A., Fan, M., Snollaerts, T., Sordoillet, V., Hamon, M., Smith, G.M., Lenkei, Z., Pezet, S., 2014. BDNF-dependent plasticity induced by peripheral inflammation in the primary sensory and the cingulate cortex triggers cold allodynia and reveals a major role for endogenous BDNF as a tuner of the affective aspect of Pain. J. Neurosci. 34, 14739–14751.
Tolle, T.R., Kaufmann, T., Siessmeier, T., Lautenbacher, S., Berthele, A., Munz, F., Zieglgansberger, W., Willoch, F., Schwaiger, M., Conrad, B., Bartenstein, P., 1999. Region-specific encoding of sensory and affective components of pain in the human brain: a positron emission tomography correlation analysis. Ann. Neurol. 45, 40–47.
Vlaeyen, J.W., Linton, S.J., 2000. Fear-avoidance and its consequences in chronic musculo-skeletal pain: a state of the art. Pain 85, 317–332.
Wu, L.J., Toyoda, H., Zhao, M.G., Lee, Y.S., Tang, J., Ko, S.W., Jia, Y.H., Shum, F.W., Zerbinatti, C.V., Bu, G., Wei, F., Xu, T.L., Muglia, L.J., Chen, Z.F., Auberson, Y.P., Kaang, B.K., Zhuo, M., 2005. Upregulation of forebrain NMDA NR2B receptors contributes to behavioral sensitization after inflammation. J. Neurosci. 25, 11107–11116.
Xiao, X., Yang, Y., Zhang, Y., Zhang, X.M., Zhao, Z.Q., Zhang, Y.Q., 2012. Estrogen in the anterior cingulate cortex contributes to pain-related aversion. Cereb. Cortex 23 (9), 2190–2203.
Xu, F., Plummer, M.R., Len, G.W., Nakazawa, T., Yamamoto, T., Black, I.B., Wu, K., 2006. Brain-derived neurotrophic factor rapidly increases NMDA receptor channel activity through Fyn-mediated phosphorylation. Brain Res. 1121, 22–34.
Yajima, Y., Narita, M., Usui, A., Kaneko, C., Miyatake, M., Narita, M., Yamaguchi, T., Tamaki, H., Wachi, H., Seyama, Y., Suzuki, T., 2005. Direct evidence for the involvement of brain-derived neurotrophic factor in the development of a neuropathic pain-like state in mice. J. Neurochem. 93, 584–594.

Yang, J.X., Hua, L., Jiang, Y.Y., Han, D., Liu, H., Tang, Q.Q., Yang, X.N., Yin, C., Hao, L.Y., Yu, L., Wu, P., Shao, C.J., Ding, H.L., Zhang, Y.M., Cao, J.L., 2015. Caveolin-1 in the anterior cingulate cortex modulates chronic neuropathic pain via regulation of NMDA receptor 2B subunit. J. Neurosci. 35 (1), 36–52.

Ying, S.W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V.P., Bramham, C.R., 2002. Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of arc synthesis. J. Neurosci. 22, 1532–1540.

Zhuo, M., 2008. Cortical excitation and chronic pain. Trends Neurosci. 31 (4), 199–207.

Zhuo, M., 2009. Plasticity of NMDA receptor NR2B subunit in memory and chronic pain. Mol. Brain 2 (4), 1–11.

Zhuo, M., 2016. Neural mechanisms underlying anxiety-chronic pain interactions. Trends Neurosci. 39 (3), 136–145.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.