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Research report

Contribution of BDNF/TrkB signalling in the rACC to the development of pain-related aversion via activation of ERK in rats with spared nerve injury

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ABSTRACT

The rostral anterior cingulate cortex (rACC) is a key structure in mediating the negative affective component of chronic pain. Brain-derived neurotrophic factor (BDNF) is known to play a critical role in activity-dependent synaptic plasticity, learning and memory. It has been shown that BDNF signalling in the rACC might be involved in spontaneous pain-related aversion, but its underlying mechanism is still largely unknown. To address this question, we measured the mRNA and protein levels of BDNF in the rACC after nerve injury and found that BDNF expression was markedly higher in nerve-injured rats than in controls. Moreover, we found that conditioned place avoidance (CPA), a behavioural phenotype reflecting pain-related aversion, was acquired in rats with partial sciatic nerve transection. However, a local injection of a BDNF-tropomyosin receptor kinase B (TrkB) antagonist into the rACC completely suppressed this process. Importantly, we found that administration of exogenous BDNF into the rACC of intact rats was sufficient to produce CPA, while selectively blocking phosphorylated extracellular signal regulated kinase (p-ERK) with a mitogen-activated protein kinase (MAPK) inhibitor U0126 completely abolished the acquisition of BDNF-induced CPA. In conclusion, we demonstrate, for the first time, that ERK is an important downstream effector of the BDNF/TrkB-mediated signalling pathway in the rACC that contributes to the development of neuropathic pain-related aversion.

1. Introduction

Neuropathic pain resulting from peripheral nerve injury is a serious clinical problem that is clinically expressed as allodynia and hyperalgesia. In fact, patients with persistent pain frequently suffer from a series of aversive emotions including anxiety, fear and depression, which are more distressing than the pain itself (Waddell et al., 1993; Crombez et al., 1999), Thus, increasing attention should be paid to the research on the mechanisms underlying the affective aspects of chronic pain. Although the emotional component of chronic pain is difficult to measure in animals and its pathogenesis is still poorly understood, the desire to avoid pain is a clear indication of the affective response to nociceptive stimulation of tissue. Thus, a behavioural testing paradigm using conditioned place avoidance (CPA) training to reflect pain-related aversion and aversive learning was established (Bravo et al., 2012).

It is well acknowledged that the rACC is a key structure in the processing of affective pain (Price, 2000). Destruction of rACC neurons in experimental rats totally blocked the acquisition of formalin-induced conditioned place avoidance (F-CPA) (Johansen and Fields, 2004), which mirrored the acute pain-related negative affect. Moreover, in patients with chronic pain, a surgical lesion of the area of the rACC alleviated the pain-related

Abbreviations: BDNF, brain-derived neurotrophic factor; MS-CPA, mechanical stimulation-induced conditioned place avoidance; CTX-B, cyclotraxin-B; ERK, extracellular signal-regulated kinase; F-CPA, formalin-induced conditioned place avoidance; MAPK, mitogen-activated protein kinase; p-ERK, phosphorylated extracellular signal regulated kinase; PFC, prefrontal cortex; rACC, rostral anterior cingulate cortex; SNI, spare nerve injury; TrkB, tropomyosin receptor kinase B.

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unpleasantness/depression (Fields and White, 1962). However, the underlying molecular mechanism of how neuropathic pain-related aversive states arise in the rACC is still unclear.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of proteins, is widely distributed in the pain-related pathway and limbic system of the central nervous system, suggesting that it is likely to play a crucial role in pain-related functions (Phillips et al., 1990; McAllister et al., 1999). Numerous studies have reported that peripheral nerve injury upregulates BDNF expression in the DRG (Kobayashi et al., 2008) as well as in the spinal dorsal horn (Geng et al., 2010), and BDNF heterozygous mutant mice have shown a marked alleviation of neuropathic pain (Yajima et al., 2005). Moreover, it is well established that the effects of BDNF are mediated through binding to its high affinity receptor TrkB and subsequently activating downstream signalling pathways (Huang and Reichardt, 2003). It was reported that neuropathic pain was prevented or attenuated by the intrathecal administration of a BDNF-sequestering TrkB-Fc fusion protein (Fukuoka et al., 2001). Although a large number of studies have focused on the generation of persistent pain mediated by BDNF/TrkB signalling, knowledge of the role of this signalling in the rACC in relation to the development of neuropathic pain-related aversion is still largely incomplete.

Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, is activated by neuronal activity and is involved in neuronal plasticity, such as long-term potentiation, learning, and memory (Wei and Zhuo, 2008; Impey et al., 1999; Sweatt, 2001). Recently, accumulating evidence has shown that ERK could be activated in the rACC after inflammation or nerve injury. Cao et al. have reported that formalin-induced inflammatory pain significantly increased ERK phosphorylation in the rACC, and this persistent upregulation of p-ERK contributed to the induction and expression of pain affect (Cao et al., 2009). Moreover, elevated levels of p-ERK expression in the rACC were also observed in a model of sciatic nerve injury, and ERK played a critical role during thermal hyperalgesia and tactile allodynia (Wei and Zhuo, 2008). Previously, we have demonstrated that BDNF/TrkB signaling participates in spontaneous pain-related aversion via NR2B receptors (Zhang et al., 2016). It was recently reported that the increased number of pERK-positive cells in the rACC of inflammatory rats could be completely suppressed by an administration of a TrkB receptor antagonist (Thibault et al., 2014). Therefore, in this study, we aim to investigate whether this BDNF signalling in the rACC contributes to the formation of pain-related negative emotions via activation of ERK in rats with peripheral nerve injury.

2. Results

2.1. Peripheral nerve injury increases BDNF expression in the rACC

First, mechanical allodynia, a behavioural sign of neuropathic pain, was measured by paw withdrawal threshold (PWT) after surgery. Compared with that in the controls, the PWT was significantly decreased in surgical rats from day 1 postsurgery to day 14 and trended to be stable on day 7 after operation (Fig. 1A). Thereafter, the mRNA level of BDNF in the rACC was measured by real-time PCR (Fig. 1B). Compared with that in the control groups, the mRNA level for BDNF was markedly increased in the rACC of surgical rats. Moreover, to further investigate the protein level of BDNF in the rACC, ELISA was performed (Fig. 1D). Compared with that in the sham-operated group, the ELISA titration of the BDNF level revealed a notable increase in the rACC of rats with neuropathic pain produced by SNI surgery. Importantly, the real-time PCR and ELISA results showed that there was no

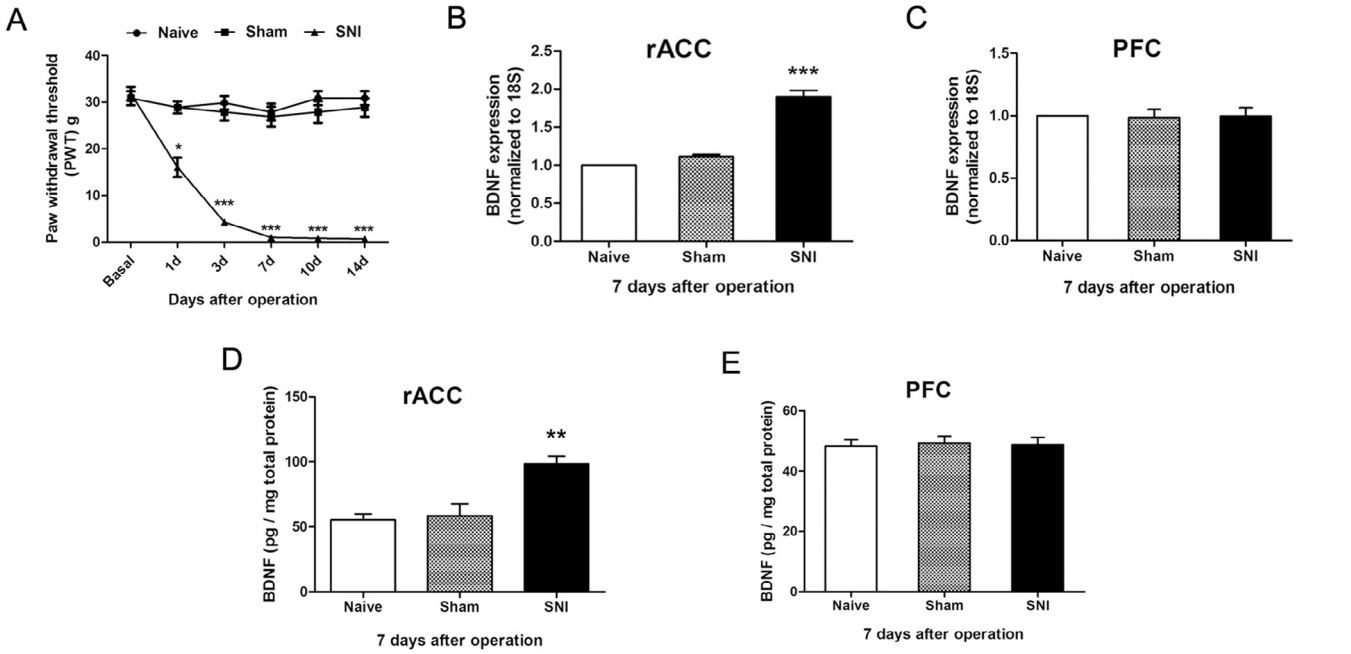

Fig. 1. The decreased threshold of mechanical allodynia and the increased concentration of BDNF in the rACC of SNI rats. (A) Mechanical allodynia measured by PWT of the injured (ipsilateral) hind paw in SNI, sham-operated and naive rats. Only SNI rats showed a significant and stable decrease in the PWT 7 days after operation compared with those in controls. $^{***}P < 0.001$, compared with pre-operation control, two-way ANOVA, $F_{(5,1,5/84)} = (28.44, 492.3, 41.27)$, n = 8/group. (B-C) The mRNA levels of BDNF in the rACC and PFC were quantified by real-time polymerase chain reaction 7 days after surgery. The mRNA expression of BDNF in the rACC was significantly higher in SNI animals than in controls. $^{***}P < 0.001$, compared with controls, one-way ANOVA, $F_{(2,15)} = 100.4$, n = 6/group. However, the mRNA level of BDNF in the PFC showed no changes. $P > 0.05$, one-way ANOVA, $F_{(2,33)} = 0.98$, n = 12/group. (D–E) The protein levels of BDNF in the rACC and PFC were detected by ELISA. The results showed that SNI significantly increased BDNF expression in the rACC. $^{***}P < 0.01$, compared with controls, one-way ANOVA, $F_{(2,6)} = 11.87$, n = 3/group. Similarly, there were no changes in the PFC region. $P > 0.05$, one-way ANOVA, $F_{(2,15)} = 0.97$, n = 6/group. Data are expressed as the mean ± SEM.

significant change in the expression of BDNF within the PFC region of SNI rats compared with that in the controls (Fig. 1C and E).

2.2. The BDNF/TrkB pathway contributes to CPA acquisition

Subsequently, the mechanical stimulus-induced conditioned place avoidance (MS-CPA) behavioural test was conducted on post-operative day 7 when the mechanical allodynia became clear and stable (Fig. 2A). The results showed that surgical, sham-operated and naive groups spent an equivalent amount of preconditioning time in the two conditioning chambers, indicating that there was no preconditioning bias for each group (Data not show). However, when a repeated MS was paired with a particular compartment in the place conditioning apparatus, the surgical rats spent significantly less time in this compartment on the post-conditioning day than on the pre-conditioning day (Fig. 2B). Sham-operated animals that received the same training did not exhibit CPA. The difference in the CPA score (i.e., the time spent in the MS-paired compartment on the pre-conditioning day minus that on the post-conditioning day) between the surgical and sham-operated rats was statistically significant (Fig. 2C).

To investigate whether and how BDNF participates in the acquisition of CPA, we determined the expression of its high affinity receptor TrkB via real-time PCR. Compared with that of the sham-operated group, the results showed that there was an increase in the mRNA level of TrkB in the rACC of rats with

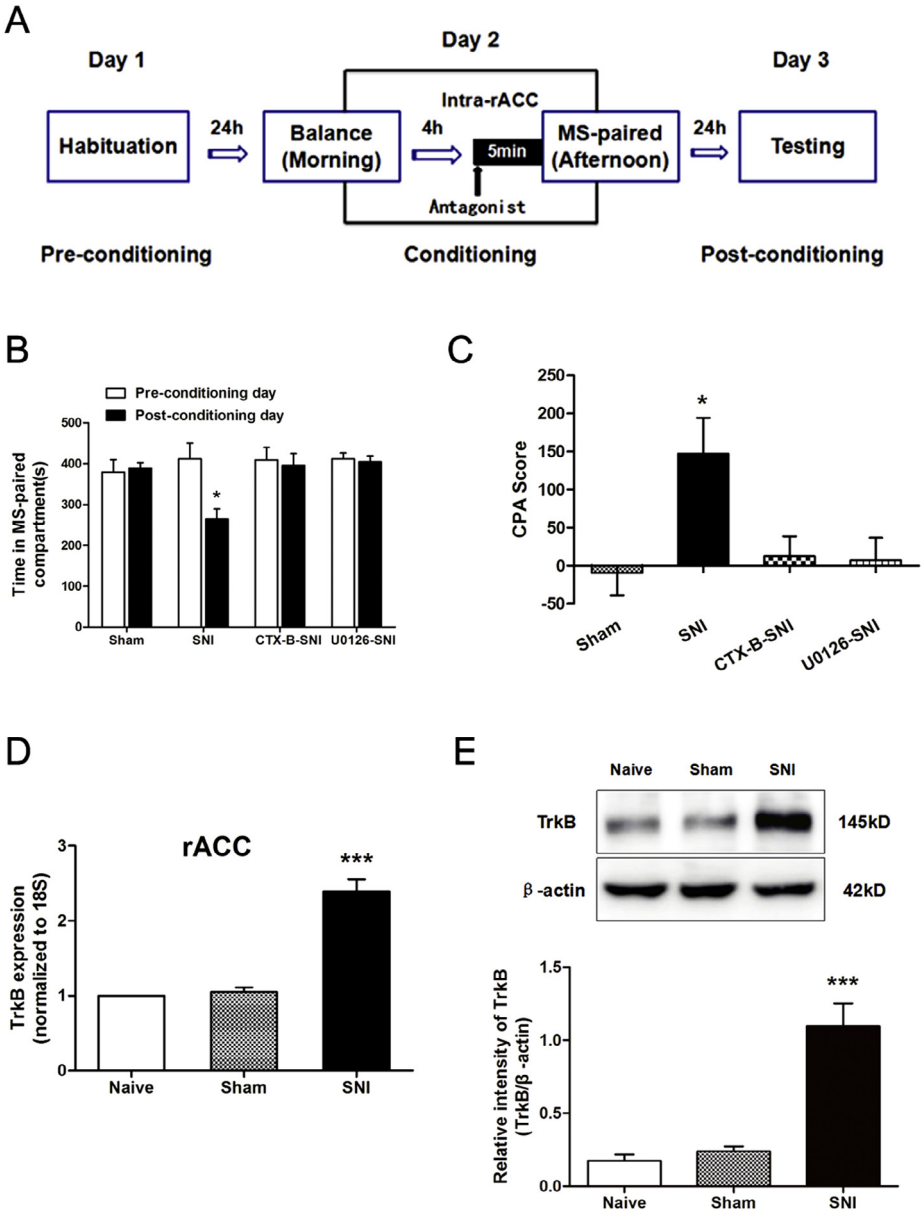

Fig. 2. BDNF/TrkB signalling participates in CPA development. (A) Behavioural test procedure (MS-CPA). (B) Only surgical rats showed a clear avoidance for the chamber paired with the mechanical stimulus. $^{*}P < 0.05$, compared with pre-conditioning, two-way ANOVA, $F_{(3,1,3/40)} = (3.845, 4.545, 2.960)$, n = 6/group. (C) The CPA score, calculated as the time spent in the mechanical stimulus-paired chamber on the pre-conditioning day minus that on the post-conditioning day, indicates that only nerve-injured rats decreased the time spent in the MS-paired compartment. However, pretreatment with CTX-B or U0126 in SNI rats completely blocked CPA acquisition. $^{*}P < 0.05$, one-way ANOVA, $F_{(3,20)} = 4.521$, n = 6/group. (D) The mRNA levels of TrkB in the rACC were quantified by real-time PCR. The mRNA expression of TrkB was significantly higher in SNI animals than in controls. $^{***}P < 0.001$, compared with controls, one-way ANOVA, $F_{(2,15)} = 64.24$, n = 6/group. (E) Western blot of TrkB expression in the rACC in naive, sham-operated, and SNI rats. Upper: representative western blot bands; lower: analysis of the relative intensity of TrkB. TrkB receptor expression was significantly higher in SNI rats than in naive and sham-operated rats. $^{***}P < 0.001$, one-way ANOVA, $F_{(2,15)} = 29.75$, n = 6/group. Data are expressed as the mean ± SEM.

neuropathic pain (Fig. 2D). In addition, western blot was carried out to determine the protein level of TrkB, and the results showed that TrkB was significantly higher in surgical rats than in the control groups (Fig. 2E). To further validate whether BDNF/TrkB-mediated signalling contributes to the development of affective pain, the highly selective TrkB receptor antagonist CTX-B (10 μg/μL, 0.5 μL per side) (Cazorla et al., 2010) was bilaterally microinjected into the rACC 5 min before MS-paired conditioning, and

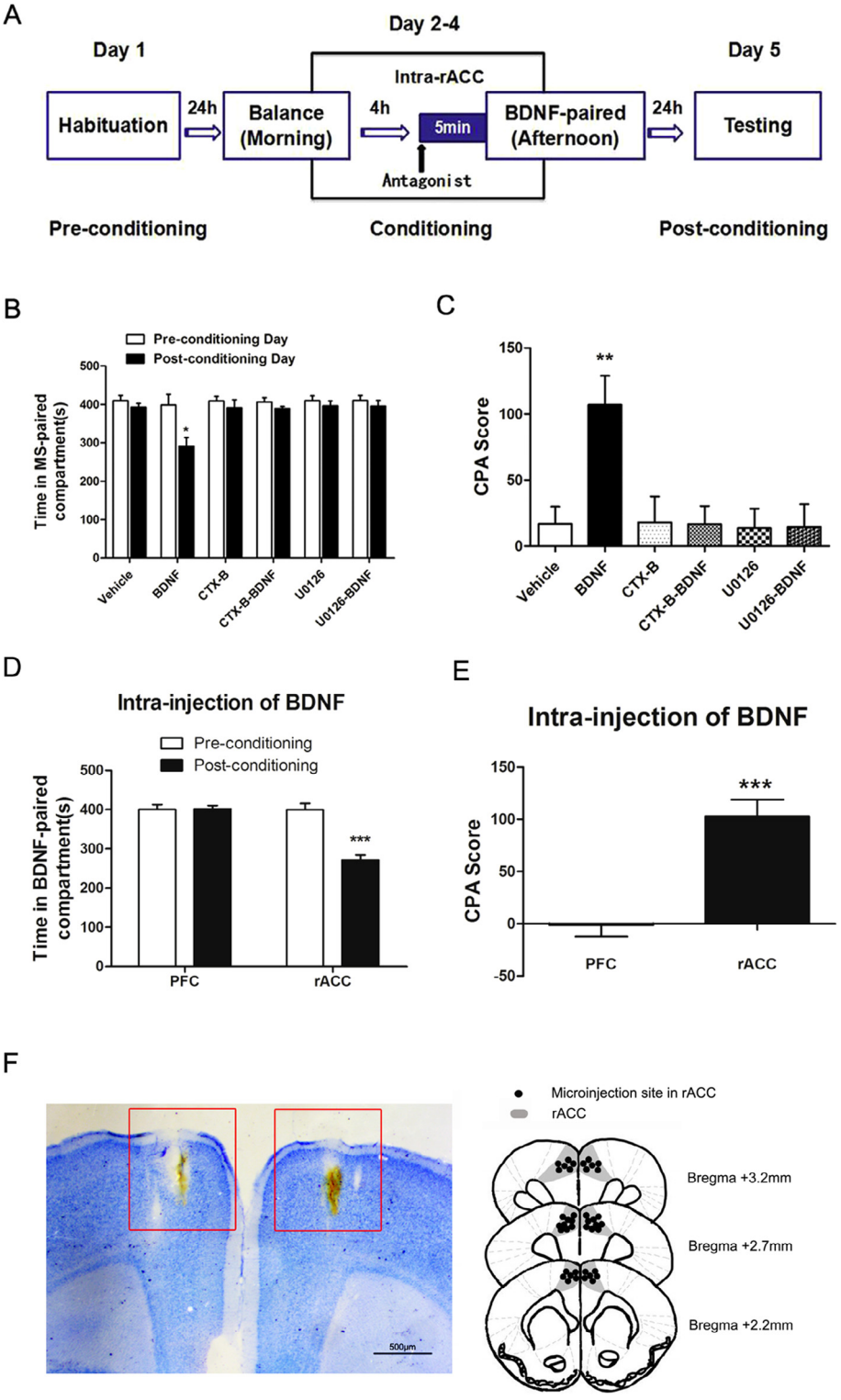

Fig. 3. Exogenous BDNF elicits CPA. (A) Schematic of the protocol for the experiments in B-C. (B) Note that only BDNF-treated rats exhibited a clear avoidance for the chamber paired with the mechanical stimulus. $^{*}P < 0.05$, compared with pre-conditioning, two-way ANOVA, $F_{(5,1,5/60)} = (2.785, 11.71, 4.307)$, n = 6/group. (C) The CPA score indicates that application of BDNF in intact rats decreased the time spent in the mechanical stimulus-paired chamber, while pre-injection of CTX-B or U0126 into the rACC attenuated the CPA score. Moreover, the intra-injection of CTX-B or U0126 alone did not produce CPA. $^{*}P < 0.01$, one-way ANOVA, $F_{(5,30)} = 4.756$, n = 6/group. (D-E) Administration of BDNF into the rACC rather than the PFC could induce CPA in naive rats. $^{***}P < 0.001$, compared with pre-conditioning, two-way ANOVA, $F(1,1,1/44) = (26.17, 24.88, 26.93)$, n = 12/group; $^{***}P < 0.001$, unpaired *t*-test, t = 5.312. n = 12/group. Data are expressed as the mean ± SEM. (F) The location of cannula manipulation and drug injection sites, scale bar = 500 μm.

the results showed that the CPA was completely blocked (Fig. 2B). However, when CTX-B was administered into the PFC instead of the rACC, CPA was not inhibited, indicating that BDNF within the rACC was likely involved in the pain-related negative affect via activation of TrkB receptors.

2.3. Exogenous BDNF elicits CPA by activating TrkB

To further investigate whether BDNF participates in pain-related aversion, exogenous BDNF (1 μg/μL, 0.5 μL per side) (Thibault et al., 2014) and an equivalent of vehicle (1% DMSO, 0.5 μL per side) as a control were administered into the rACC of intact rats (Fig. 3A). Interestingly, we acquired a BDNF-induced CPA when direct injection of BDNF into the rACC was paired with the conditioning chamber. The results showed that rats with BDNF application spent significantly less time in the BDNF-paired compartment on the post-conditioning day than on the pre-conditioning day, while there were no changes in vehicle-treated rats (Fig. 3B). The CPA score was significantly increased in rats pre-treated with BDNF (Fig. 3C). However, this avoidance phenomenon disappeared when BDNF was administered into the area of the PFC (Fig. 3D–E). Furthermore, we also found that the BDNF-induced CPA was completely prevented by pre-injection of a TrkB antagonist CTX-B into the rACC, and intra-rACC injection of CTX-B alone did not produce CPA (Fig. 3B). Additionally, the location of cannula manipulation and drug injection in rACC were identified by cresyl violet staining (Fig. 3F).

2.4. ERK is activated by BDNF-mediated signalling

Phosphorylation of ERK has been extensively used as an indicator of ERK activation (English and Sweatt, 1996; Ji et al., 1999). Thus, immunohistochemistry was performed to determine the p-ERK expression in the rACC, and the results showed that the expression of the p-ERK protein was significantly increased in surgical rats compared with the naive and sham-operated groups (Fig. 4A). To investigate whether p-ERK participated in CPA acquisition, U0126, a highly selective inhibitor of MAPK that blocks phosphorylation of ERKs, was pre-injected into the rACC of SNI rats before conditioning. As we expected, CPA was completely prevented (Fig. 2B), which suggested that p-ERK is likely to play a crucial role in CPA development. At the same time, the western blot (Fig. 4B) and immunofluorescence (Fig. 5) results showed that the upregulation of p-ERK was almost completely blocked by application of U0126.

Several studies have revealed that ERK could be activated by the TrkB receptor (Klein et al., 1991; Thibault et al., 2014). Therefore, we aimed to determine the potential association between BDNF/TrkB signalling and the ERK protein. The results of the western blot showed that SNI surgery dramatically increased the p-ERK expression compared with that in control groups. However, the upregulation was mostly suppressed by an intra-rACC administration of the TrkB receptor antagonist CTX-B (Fig. 6A), which indicated that the expression of p-ERK was likely regulated by BDNF/TrkB-mediated signalling.

2.5. ERK activation in the rACC is required for the development of pain affect

To further validate that the activation of ERK was indispensable for the BDNF signalling-induced pain affect, the selective p-ERK inhibitor U0126 (2 nmol, 0.5 μL per side) (Cao et al., 2009) was locally injected into the rACC before BDNF-paired conditioning. In accordance with our hypothesis, BDNF-induced CPA was mostly

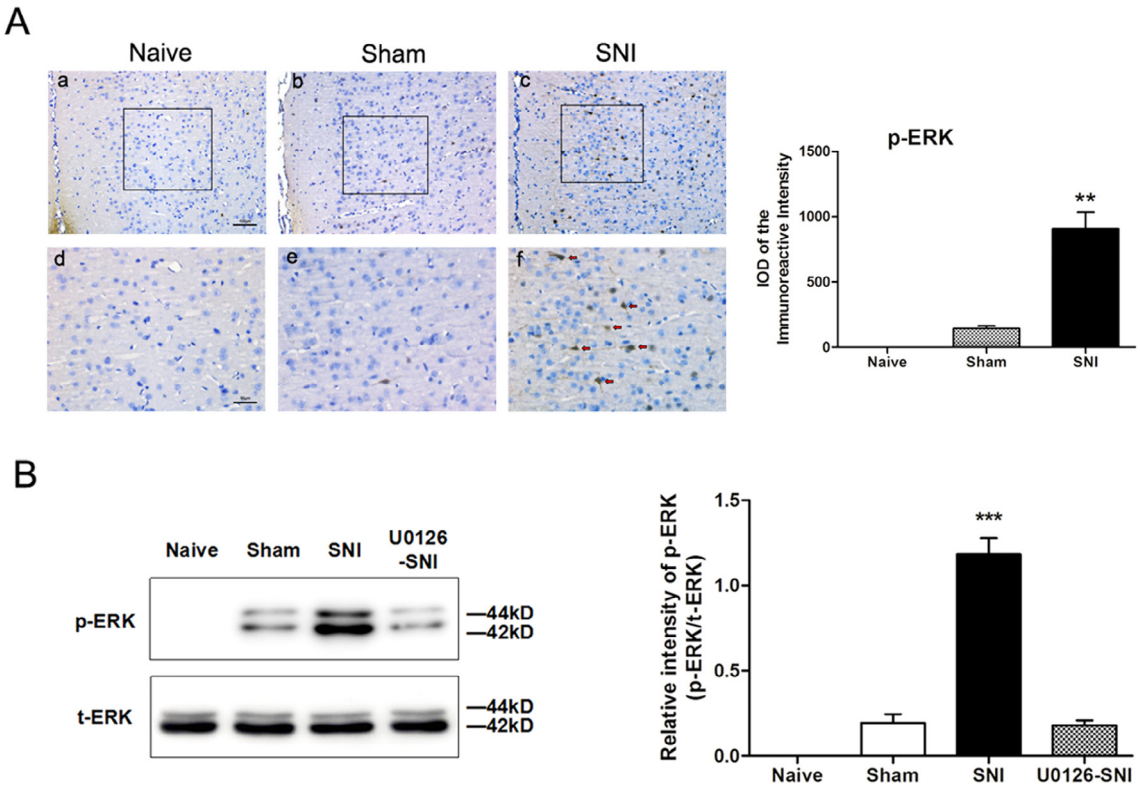

Fig. 4. ERK is activated after nerve injury. (A) Expression of p-ERK in the rACC. Immunohistochemical staining under 20× (a-c, scale bar = 100 μm) and 40× (d-f, scale bar = 50 μm) objective showed that p-ERK in the rACC was significantly increased in SNI rats but not in sham-operated or naive rats. $^{**}P < 0.01$, compared with controls, one-way ANOVA, $F_{(2,12)} = 45.40$, n = 5/group. (B) Western blot of p-ERK expression in the rACC in naive, sham-operated, and SNI rats and rats pre-treated with U0126. Total-ERK was used as internal control. The results indicate that expression of p-ERK was significantly higher in SNI rats than in the control groups, but pretreatment with U0126 almost completely blocked the upregulation of p-ERK. $^{***}P < 0.001$, one-way ANOVA, $F_{(3,20)} = 90.50$, n = 6/group. Data are expressed as the mean ± SEM.

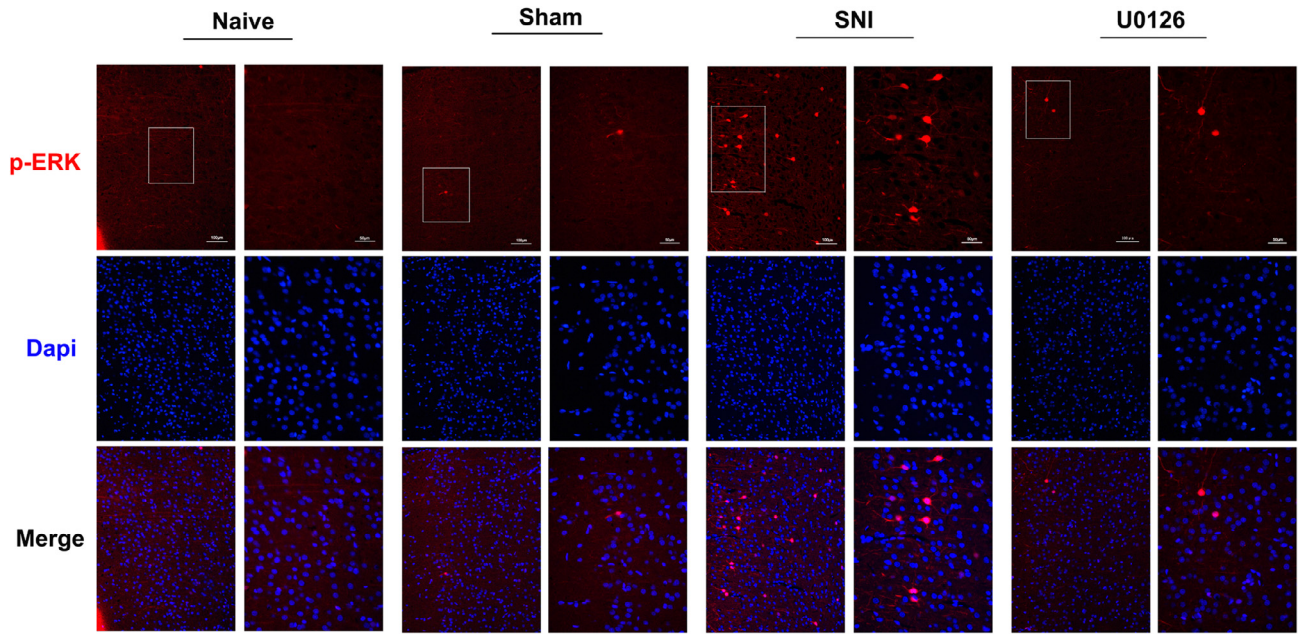

Fig. 5. Immunofluorescence showed the expression of p-ERK (red) in the rACC of different groups under 20× (Scale bar = 100 μm) and 40× (Scale bar = 50 μm) objectives. The results showed that SNI surgery dramatically increased the p-ERK expression compared with that in control groups. However, the upregulation was mostly suppressed by an intra-rACC administration of the p-ERK antagonist U0126.

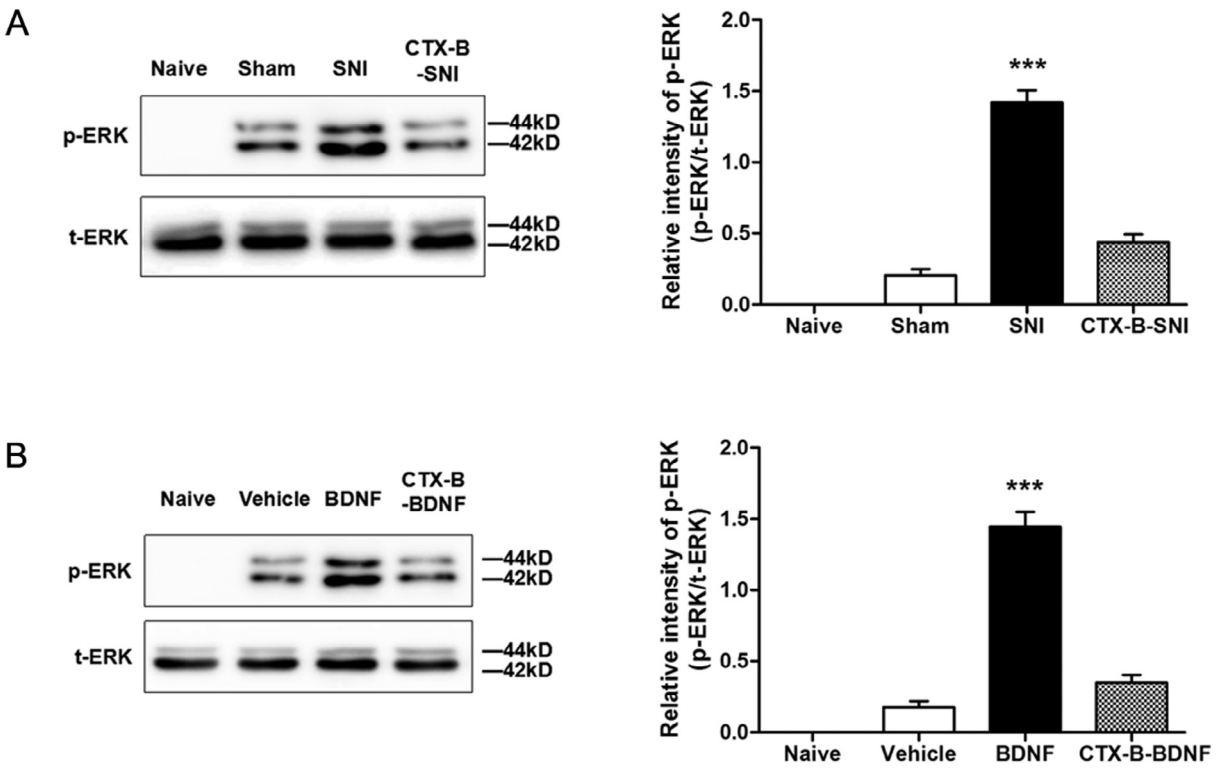

Fig. 6. SNI surgery or administration of exogenous BDNF into the rACC in intact rats significantly increases p-ERK expression in the rACC, while pretreatment with CTX-B prevents the upregulation of p-ERK. (A) Western blot of p-ERK expression in the rACC in naive, sham-operated, and SNI rats and SNI rats pre-treated with CTX-B. Total-ERK was used as an internal control. The expression of p-ERK was prominently higher in SNI rats than in naive and sham-operated rats. However, this upregulation was mostly blocked by preemptive application of CTX-B. $^{***}P < 0.001$, one-way ANOVA, $F_{(3,20)} = 130.8$, n = 6/group. (B) Western blot of NR2B expression in the rACC in naive, vehicle-treated, and BDNF-treated rats and rats pre-treated with CTX-B. Total-ERK was used as an internal control. Expression of p-ERK was significantly higher in BDNF-treated rats than in naive and vehicle-treated rats, but pretreatment with CTX-B alleviated the upregulation of p-ERK. $^{***}P < 0.001$, one-way ANOVA, $F_{(3,20)} = 108.7$, n = 6/group. Data are expressed as the mean ± SEM.

blocked by application of U0126 (Fig. 3B). To provide further evidence for our hypothesis that increased BDNF in the rACC activates p-ERK, which subsequently induces a pain-like aversive state, we measured the expression of p-ERK protein in the rACC. BDNF was delivered to intact rats as described in the aforementioned behavioural experiments, and subsequently, western blot analysis was

performed. The results showed that the p-ERK expression was significantly increased by BDNF in the rACC following BDNF-induced CPA conditioning. However, when CTX-B, the highly potent and selective TrkB inhibitor, was administered into the rACC before application of BDNF, the upregulation of p-ERK was mostly suppressed, which suggested that p-ERK expression was regulated through the BDNF/TrkB-related pathway (Fig. 6B).

3. Discussion

This study investigates the putative role of BDNF signalling in mediating neuropathic pain-related negative emotion. Peripheral nerve injury induced a significant upregulation of BDNF in the rACC and subsequently produced CPA. However, blocking the BDNF signalling pathway by administration of a TrkB receptor antagonist completely prevented MS-CPA acquisition. Furthermore, application of exogenous BDNF in normal rats was sufficient to produce CPA, and this process was completely blocked by a pre-injection of a p-ERK inhibitor into the rACC. In the current study, we present valid evidence demonstrating that BDNF/TrkB signalling-induced ERK activation in the rACC is required for acquisition of CPA behaviour, suggesting that this pathway contributes to the development of neuropathic pain-related aversion following peripheral nerve injury.

Neuropathic pain is one of the most common types of chronic pain in clinical practice and is usually accompanied with emotional disturbance. It is easy to investigate the affective (unpleasant) component of persistent pain in human studies, such as worry, fear, etc. (Melzack, 1975). However, precisely defining the unpleasantness in animal studies is difficult, and the complex paradigms often used involve cognitive-affective components that, in turn, may create data bias. Therefore, a nociceptive stimulus-induced CPA paradigm has been used to evaluate supraspinal areas involved in pain-related aversion (LaBuda and Fuchs, 2000). In the current study, when mechanical stimulation, an aversive/unpleasant signalling maintained in memory, was paired with a specific conditioned compartment, CPA was successfully produced. Moreover, we found that CPA was only acquired in surgical rats but not in sham-operated or naive groups, which suggested that peripheral nerve injury caused a pain-related negative emotion. The CPA behavioural test was further shown to be an effective approach to investigate pain-related negative affection.

Accumulating evidence suggests an important role of the rACC in pain-related negative affect (Johansen et al., 2001; Kung et al., 2003; Gao et al., 2004). A large number of preclinical studies have found that lesion of the rACC prevented F-CPA (formalin-induced conditioned place avoidance) without affecting formalin-induced acute pain-related behaviours, such as lifting, licking, and flinching the stimulated paw (Johansen et al., 2001). Donahue et al. and LaGraize et al. demonstrated that rACC lesions prevent avoidance learning in nerve-injured rats without altering mechanical hyperalgesia (Donahue et al., 2001; LaGraize et al., 2004). In the present study, we have proven that pre-injection with the TrkB receptor antagonist CTX-B in the rACC completely blocked MS-CPA, while intra-PFC injection of CTX-B failed to prevent CPA. Moreover, the direct administration of exogenous BDNF into the rACC rather than the PFC produced CPA, suggesting a crucial role of the rACC in BDNF signalling-mediated aversive learning. All of these results further demonstrated that the rACC plays a critical role in mediating the negative emotional component of chronic pain.

BDNF is widely distributed in the sensory and limbic systems and is essential for maintenance of the related functions of pain, memory, and depression/anxiety (Li and Li, 2015; Merighi et al., 2008). It was previously reported that both the concentration and protein expression of BDNF in the spinal cord significantly increases in rats following nerve injury (Geng et al., 2010; Miletic and Miletic, 2002). Compelling evidence has shown that BDNF is involved in spinal plasticity and central sensitization and, therefore, plays an important role in the development of persistent pain (Garraway et al., 2003; Marcol et al., 2007). In addition, it is well acknowledged that BDNF exerts its effects via its high affinity receptor TrkB, and the BDNF/TrkB signalling pathway is likely involved in synaptic mechanisms underlying both memory and pain (Merighi et al., 2008). Thus, we measured the BDNF and TrkB expression in the rACC and found that SNI surgery markedly upregulated the expression of these two proteins, suggesting that increased BDNF participated in neuropathic pain formation. Subsequently, we investigated whether BDNF signalling in the rACC played a critical role in the development of neuropathic pain-related aversion in rats that suffered from nerve injury. The findings showed that CPA was completely blocked by pre-application of a TrkB receptor antagonist into the rACC. Moreover, we also found that administration of exogenous BDNF in normal rats was sufficient to induce CPA behaviour, suggesting that increased expression of BDNF in the rACC potentiates the negative emotional aspect of chronic neuropathic pain. However, when CTX-B, a highly selective TrkB receptor antagonist, was pre-injected into the rACC before conditioning, CPA acquisition was completely prevented. Compared with our previous study (Zhang et al., 2016), we further confirmed the contribution of increased BDNF/TrkB-mediated signalling in the rACC to the pathogenesis of pain-related aversion in rats suffering from nerve injury.

ERK phosphorylation has been widely used as an indicator of ERK activation (English and Sweatt, 1996). ERK can be activated by neural excitation, and there is considerable evidence showing the involvement of p-ERK in pain hypersensitivity (Dai et al., 2011). The molecular mechanisms underlying ERK-mediated pain hypersensitivity have been examined, but findings in discrete brain regions related to pain emotion have not been well established. ERK activation in the rACC may serve as a marker for the activation of rACC neurons in an affective pain condition (Cao et al., 2009). In addition, ERK activation in the rACC was required for the development of affective pain, as intra-rACC application of a MEK inhibitor, PD98059 or U0126, prevented the induction of F-CPA (Cao et al., 2009). In accordance with the above findings, our study demonstrated that peripheral nerve injury induced a significant upregulation of p-ERK in the rACC, while MS-CPA was completely blocked by pre-administration of a selective MEK inhibitor U0126 into the rACC, further indicating that activation of ERK in the rACC is required for the expression of pain-related aversion. Moreover, it was reported that ERK could be activated by BDNF. For example, administration of exogenous BDNF induced a rapid activation of ERK in the spinal cord, while sequestering BDNF with a TrkB-IgG fusion protein significantly blocked the activation of ERK evoked by noxious stimulation (Pezet et al., 2002; Lever et al., 2003). Obata et al. found that CFA-induced inflammation produced an increase in the phosphorylation of ERK, while intrathecal treatment with the TrkB inhibitor reduced the CFA-induced increase in ERK phosphorylation (Obata et al., 2004). In the current study, we provided biochemical evidence that ERK was significantly activated in SNI rats. However, when we blocked the BDNF-related signalling by TrkB receptor antagonist CTX-B, the increased expression of p-ERK was mostly suppressed. This result suggested that BDNF might play a key role in the activation of ERK following SNI surgery. Thereafter, we found that application of exogenous BDNF induced a significant increase in p-ERK expression, while the upregulation of p-ERK was mostly prevented by CTX-B, which further indicated that BDNF/TrkB signalling contributed to the development of pain-related aversion by activating the ERK.

To our knowledge, this is the first study to show that enhanced BDNF expression in the rACC participates in the aversive state of

neuropathic pain, and activated ERK is an indispensable downstream effector of the BDNF/TrkB signalling pathway. This research is clinically significant and suggests that a new strategy targeting BDNF-related signalling in the rACC is likely to be useful for the alleviation of pain-related emotional disturbance due to peripheral nerve injury.

4. Experimental procedure

4.1. Animals

Male Sprague-Dawley rats weighing 200–220 g at the beginning of the experiments were obtained from the Experimental Animal Center of Shandong University. Rats were housed in separate cages with free access to food and water. They were on a 12:12 light/dark cycle with a room temperature of 23 ± 1 °C and a humidity of 40–50%. All experimental protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Shandong University.

4.2. Spare nerve injury (SNI) surgery

Spare nerve injury models were established as previously described (Decosterd and Woolf, 2000). Briefly, rats were anesthetized by intraperitoneal injection of chloral hydrate (40 mg/kg). The sciatic nerve and its three terminal branches, the tibial, common peroneal and sural nerves, were exposed by incising the skin on the lateral surface of the thigh and separating the biceps femoris muscle. The SNI surgery comprised a ligation and axotomy of the tibial and common peroneal nerves. The tibial and common peroneal nerves were ligated tightly with a 5.0 silk suture and sectioned distal to the ligation, removing 2–4 mm of the distal nerve stump. Great care was taken to avoid any stretching of the intact sural nerve. The sham-operated group received the same surgical procedure without the sectioning of the tibial and common peroneal nerves.

4.3. Assessment of mechanical allodynia

Mechanical allodynia, as a behavioural sign of neuropathic pain, 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 as previously described (Berrocoso et al., 2011). Animals were allowed to adapt to the testing environment for 30 min. A series of 12 standardized von Frey hairs were applied to the plantar surface of one hind paw in ascending order ranging from 0.38 to 34.8 g. A valid withdrawal response was indicated by the complete removal of the hind paw 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 1, 3, 7, 10 and 14 days after surgery.

4.4. rACC cannulation and microinjections

Rats were anesthetized with chloral hydrate and securely placed into a stereotaxic device with Bregma and Lambda at a horizontal level. A 30-gauge stainless steel guide cannula was implanted into the bilateral rACC [anteroposterior (AP) + 2.6 from bregma, mediolateral (ML) ± 0.6, dorsoventral (DV)−2.5] 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] according to the atlas of Paxinos and Watson (1998) (Paxinos and Watson, 1998). The cannula was attached to the bone with stainless steel screws and acrylic cement. A 33-gauge stainless steel wire was inserted into the guide cannula to prevent blocking. The rats were allowed to recover for at least 7 days before the next experimental procedure. The brain slice with cresyl violet staining was used to verify the location of cannula manipulation and drug injection.

Microinjection was performed through a 33-gauge stainless-steel injection cannula that was connected to a 1-μL Hamilton syringe with PE-10 tubing. BDNF (1 μg/μL, 0.5 μL per side, R&D Systems), CTX-B (10 μg/μL, 0.5 μL per side, R&D Systems) or vehicle [0.5 μL per side, 1% dimethylsulfoxide (DMSO)] was administered over a 5-min period. Following the completion of the drug infusion, the injection cannula was left in place for an additional 5-min to minimize the drugs leaking out through the injection track. All behavioural tests were performed blind with respect to the drug injections.

4.5. Nissl (Cresyl violet) staining

To verify the injection site of rACC, a brain slice was cut for Nissl staining in rats with intra-rACC injection of drugs. The parafin sections were immersed in cresyl violet staining solution at 60 °C for 40 min. The sections were dehydrated in an ascending series of ethanol. Finally, they were observed and photographed with a Leica SP2 light microscope (Olympus, Japan).

4.6. Behavioural studies – conditioned place avoidance

The CPA procedure was performed as previously described (Bravo et al., 2012) with slight modifications. The place conditioning apparatus was comprised of two large conditioning chambers (30 × 30 × 30 cm, length × width × height) with distinct olfactory, visual, and tactile cues and a smaller neutral-choice chamber (15 × 20 × 30 cm). Briefly, one of the conditioning compartments is made of black and white vertical stripe, and the bottom is made of a rough plastic sheet with drops of acetic acid as an odor agent. The other conditioning room is made of black and white cross stripe, and the bottom is made of a smooth plastic sheet with drops of cinnamon water as an odor agent. The smaller neutral-choice chamber wall is grey, with no special sign or special odor. The MS-CPA (mechanical stimulation-induced conditioned place avoidance) task required three days. Day 1 was the pre-conditioning day. At the beginning, a rat was placed in the neutral compartment. After habituating for 2 min, the rat was allowed to explore the two conditioning compartments freely for 15 min. A timer automatically recorded the time spent in each of the compartments. Rats that spent >80% (720 s) on one side on day 1 were excluded from the subsequent experiments. Day 2 was the conditioning day. On this day, the plantar surface of the noninjured (contralateral) hind paw was mechanically stimulated at 3-min intervals with a Von Frey filament (60 g) in one of the conditioning chambers for 30 min. After at least 4 h, the rat was kept in the other conditioning chamber for 30 min and was stimulated mechanically at 3-min intervals on the surface of the injured (ipsilateral) hind paw with a Von Frey filament (60 g). The control group received the same training. Day 3 was the post-conditioning day. The procedure was the same as day 1. The degree of conditioned place avoidance was measured by the CPA score (the time spent in the treatment-paired compartment on the pre-conditioning day minus that on the post-conditioning day).

For the BDNF-induced CPA, the procedures on the first and last day were the same as that described above. However, on the training day, no mechanical stimuli was conducted. Exogenous BDNF was directly administered into the rACC of intact rats 5 min before CPA training, and the antagonist was pre-injected into the rACC

10 min before application of BDNF. Then, the same training was repeated for another 2 days.

4.7. ELISA detection of BDNF concentration in the rACC

Animals were sacrificed by overdose of chloral hydrate (80 mg/kg). According to the coordinates in the Paxinos and Watson rat brain atlas, the brain areas that comprised the pooled bilateral rostral anterior cingulate cortex or pooled bilateral prefrontal cortex were quickly dissected and immediately frozen in liquid nitrogen. Briefly, samples were homogenized in ice-cold PBS containing 0.05% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM ethylene diamine tetraacetic acid, and 20 IU aprotinin A. Tissue samples were centrifuged for 10 min at 10000 rpm, and the supernatants were collected and stored at −80 °C for future analysis. Rat enzyme linked immunosorbent assay (ELISA) kits (R&D System, USA) were used to detect the BDNF levels, and the results were expressed in picograms BDNF/mg protein.

4.8. RNA extraction

Rats were deeply anesthetized with an overdose of chloral hydrate followed by perfusion with 200 ml saline. The brain areas that comprised the pooled bilateral rACC or pooled bilateral PFC were quickly removed, frozen immediately in liquid nitrogen, and store at −80 °C. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The extracted total RNA was eluted in 20 μL nuclease-free water, and the RNA concentration was measured by NanoDrop 2000 (Thermo Fisher Scientific).

4.9. Quantitative real-time polymerase chain reaction

For BDNF and TrkB mRNA quantification, RNA was reverse-transcribed with a random primer using a TaKaRa Reverse Transcription Kit (TaKaRa, Dalian, China). mRNA levels were determined with RT-qPCR using the SYBR Green master mix on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) with the housekeeping gene 18S as an internal control. The primer sequences were as follows: 18S-forward, 5′GCT GTG GTC CAA GGC CAT TTT 3′; 18S-reverse, 5′ CCG AGT TAC TTT TCC CCA GAT GAC 3′; BDNF-forward, 5′CAA GGC AAC TTG GCC TAC CC 3′; BDNF-reverse, 5′ GAG CAT CAC CCG GGA AGT GT 3′; TrkB-forward, 5′AAG ATC CTG GTG GCC GTG AAG A 3′; TrkB-reverse, 5′ CGG CTT CGC GAT GAA AGT CCT T 3′.

4.10. Immunohistochemistry and immunofluorescence

At the indicated time points, rats were sacrificed by overdose of chloral hydrate after behavioural studies. The brains from the control and experimental rats were fixed for 4 h with 4% paraformaldehyde after perfusion and cryoprotection by immersion in 20% sucrose in phosphate buffer (pH 7.4) 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 the antigen and 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 the primary rabbit anti-phospho-p44/42 (extracellular signal–regulated protein kinases 1 and 2 [Erk1/2]) (1:400; Cell Signaling Technology, USA) at 4 °C overnight. On the second day, all sections were incubated with biotinylated goat anti-rabbit secondary antibody at 37 °C for 30 min and then incubated with avidin-biotin-peroxidase complex at 37 °C for 30 min. Finally, the sections were allowed to react with diaminobenzidine (DAB) for 1–2 min. For relative quantification of immunoreactivity, the integrated optical density (IOD) of the immunoreactive intensity in the rACC was measured.

For p-ERK immunofluorescence, sections were incubated with rabbit anti-phospho-p44/42 (extracellular signal–regulated protein kinases 1 and 2 [Erk1/2]) (1:50; Cell Signaling Technology) overnight at 4 °C. On the second day, all of the steps were protected from the light. Sections were incubated with the FITC-conjugated secondary antibody for 30 min at 37 °C. Thereafter, the sections were incubated with DAPI for 5 min. Finally, the sections were observed and photographed in the dark room with a Leica SP2 confocal laser scanning microscope (Olympus, Japan).

4.11. Western blot analysis

After the behavioural tests, rats were sacrificed by overdose of chloral hydrate, and the brains were removed rapidly. The bilateral rACC was dissected on ice using a surgical blade according to the atlas of Paxinos and Watson followed by nitrogen quickly frozen in liquid. Proteins were extracted from the frozen samples in lysis buffer containing protease inhibitor cocktails (Roche Applied Science) and PMSF (Sigma Chemical Company). Samples were centrifuged at 10000 rpm for 30 min at 4 °C. The sections were stored at −80 °C for western blotting. Equal amounts of protein (40 μg) were loaded and separated in a Tris-Tricine SDS-PAGE gel. Then, the resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 10% non-fat milk for 2 h at room temperature and incubated with rabbit anti-TrkB (1:1000; Cell Signaling Technology, USA), rabbit anti-phospho-p44/42 (extracellular signal–regulated protein kinases 1 and 2 [Erk1/2]) and rabbit anti-p44/42 (Erk1/2) (1:500; Cell Signaling Technology, USA) primary antibody on the shaking table overnight at 4 °C. On the next day, the blots were then incubated with secondary antibody goat anti-rabbit IgG (1:5000, Cell Signaling Technology, USA) conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. Immunoblots were visualized using the Immobilon™ Western Chemiluminescent HRP Substrate (Millipore).

4.12. Drugs

The drugs used in the present study were BDNF (R&D Systems), cyclotraxin (CTX-B, R&D Systems), and a selective p-ERK inhibitor U0126 (R&D Systems). BDNF and CTX-B were dissolved in 0.9% sterile physiological saline. U0126 was dissolved in 0.9% sterile physiological saline containing 35% dimethylsulfoxide (DMSO).

4.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, USA). Data are expressed as the mean ± SEM. 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. Unpaired two-tailed Student's *t*-test was used for the comparison of two groups. A value of $P < 0.05$ was considered statistically significant.

Conflicts of interest

The authors declare there is no conflict of interest.

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