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Original Article

GluN2B-BDNF pathway in the cerebrospinal fluid-contacting nucleus mediates nerve injury-induced neuropathic pain in rats

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Abstract: The present study was aimed to investigate the role of GluN2B-BDNF pathway in the cerebrospinal fluid-contacting nucleus (CSF-CN) in neuropathic pain. Intra-lateral ventricle injection of cholera toxin subunit B conjugated with horseradish peroxidase (CB-HRP) was used to label the CSF-CN. Double-labeled immunofluorescent staining and Western blot were used to observe the expression of GluN2B and BDNF in the CSF-CN. Chronic constriction injury of sciatic nerve (CCI) rat model was used to duplicate the neuropathic pain. Pain behavior was scored to determine the analgesic effects of GluN2B antagonist Ro 25-6981 and BDNF neutralizing antibody on CCI rats. GluN2B and BDNF were expressed in the CSF-CN and their expression was up-regulated in CCI rats. Intra-lateral ventricle injection of GluN2B antagonist Ro 25-6981 or BDNF neutralizing antibody notably alleviated thermal hyperalgesia and mechanical allodynia in CCI rats. Moreover, the increased expression of BDNF protein in CCI rats was reversed by intra-lateral ventricle injection of Ro 25-6981. These results suggest that GluN2B and BDNF are expressed in the CSF-CN and alteration of GluN2B-BDNF pathway in the CSF-CN is involved in the modulation of the peripheral neuropathic pain.

Key words: cerebrospinal fluid-contacting nucleus; GluN2B; CB-HRP; BDNF; neuropathic pain

触液核GluN2B-BDNF通路介导大鼠神经病理性疼痛的发生

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摘 要：本研究旨在探讨触液核GluN2B-BDNF通路在神经病理性疼痛中的作用。应用侧脑室注射特异性触液核示踪剂霍乱毒素亚单位B与辣根过氧化物酶复合物(cholera toxin subunit B conjugated with horseradish peroxidase, CB-HRP)的方法标记触液核；通过免疫荧光双标染色和Western blot观察大鼠触液核GluN2B和BDNF的表达；采用坐骨神经慢性压迫性损伤法(chronic constriction injury of sciatic nerve, CCI)建立大鼠慢性神经病理性疼痛模型；通过侧脑室注射GluN2B拮抗剂和BDNF中和抗体观察CCI大鼠的行为学变化。结果显示，GluN2B和BDNF均在触液核内表达，并且在CCI大鼠表达上调；侧脑室注射GluN2B拮抗剂或BDNF中和抗体能够减轻CCI大鼠的热痛觉过敏和机械性痛觉超敏；而且侧脑室注射GluN2B拮抗剂能够逆转CCI大鼠BDNF的表达上调。以上结果提示，大鼠触液核内有GluN2B和BDNF的表达，并且触液核GluN2B-BDNF通路参与了大鼠神经病理性疼痛的发生。

关键词：触液核；GluN2B；CB-HRP；BDNF；神经病理性疼痛
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The distal cerebrospinal fluid-contacting neurons (dCSF-CNs) are a peculiar cell type of the central nervous system, with somata in the parenchyma of the brain and projections extending into the cerebrospinal fluid (CSF) in the cavity of the ventricle in the central nervous system [1]. With proper tracers, such as cholera toxin subunit B conjugated with horseradish peroxidase (CB-HRP), the dCSF-CNs can be easily identified from the non-CSF-CNs in the parenchyma of the brain. By injecting CB-HRP into the lateral ventricle (LV), the dCSF-CNs were found to be localized in the ventral periaqueductal gray (PAG) of the brainstem, which formed the cerebrospinal fluid-contacting nucleus (CSF-CN) [2]. Previous studies indicated that CSF-CN was involved in pain transmission and modulation in rat models of inflammatory and neuropathic pain [3, 4]. However, the cellular mechanisms underlying nerve injury-induced neuropathic pain in CSF-CN are poorly understood.

There is compelling evidence for the involvement of the NMDA receptors (NMDARs) in the development of chronic pain, in particular neuropathic pain [5–7]. Prolonged nociceptive stimulation will activate and up-regulate the NMDARs, causing enhanced and amplified trafficking of pain signals to central sites (central sensitization) [8]. NMDAR antagonists effectively reduce neuropathic pain, but serious side effects prevent their use as therapeutic drugs [9]. Functional NMDARs are heteromultimeric complexes that are comprised of at least two types of subunits, the structure subunit GluN1 and the modulatory subunit NR2A–D in the central nervous system [10]. GluN1/GluN2B NMDAR is important for the treatment of chronic pain. This receptor type is restricted to areas involved in pain signaling [11, 12]. The GluN2B plays a crucial role in central pain pathways and inhibition of this receptor by selective NMDAR antagonists may produce analgesia. Previous studies showed that intrathecal injection of GluN2B antagonist increased the mechanical nociceptive threshold after spinal cord injury without motor depression [13, 14]. Despite the presence of GluN2B receptors in the dorsal horn, the selective GluN2B antagonist did not antagonize the response of dorsal horn neurons to NMDA and did not block wind-up, which indicated that the GluN2B selective compounds had a supraspinal site of antinociceptive action [15]. Since CSF-CN plays an important role in processing pain-related information, it raises the possibility that GluN2B expression is altered in the CSF-CN after peripheral nerve injury and targeting GluN2B in the CSF-CN could be an effective way to treat neuropathic pain. Therefore, we hypothesize that CSF-CN may be one of the central locations where the selective GluN2B antagonist mediates its analgesic effect.

Previous studies indicated that brain-derived neurotrophic factor (BDNF) had a crucial role in the development of neuropathic pain [16–18]. Increased BDNF in the spinal dorsal horn was likely to be associated with the initiation of neuropathic pain. Inhibition of BDNF with anti-BDNF antibody alleviated nerve injury-induced mechanical allodynia [19]. Central sensitization induced by BDNF is a dose-independent, all-or-none process. BDNF alone is sufficient for generating a long-lasting neural excitability change in the spinal cord via tyrosine kinase B receptor signaling [20]. Studies also showed that BDNF in spinal cord contributes to the development and maintenance of neuropathic pain through the activation of GluN2B in the dorsal horn [21]. So far, there are few studies exploring whether or not BDNF plays a similar role in CSF-CN.

This research aimed to provide further insight into the mechanism of GluN2B in the CSF-CN involving in pain processing. Firstly, we examined the distribution of GluN2B in the CSF-CN and assessed the variation trend of GluN2B protein expression in the CSF-CN in chronic constriction injury (CCI) model of neuropathic pain. Thereafter, we tested the hypothesis that the analgesic effects of Ro 25-6981, a GluN2B antagonist, in CCI rats are mediated centrally in the CSF-CN. Finally, whether BDNF in the CSF-CN participates in nerve injury-induced neuropathic pain and how inhibition of GluN2B influences the alteration of BDNF were investigated. Our results demonstrate that GluN2B and BDNF are up-regulated in the CSF-CN, and GluN2B-BDNF pathway is involved in the modulation of the peripheral nerve injury.

1 MATERIALS AND METHODS

1.1 *CCI surgery*

Adult male Sprague-Dawley rats weighing 200–250 g were obtained from the experimental animal center, Xuzhou Medical College. The SPF grade rats were maintained in climate and light-controlled [(23 ± 1) °C, 12 h/12 h dark/light cycle with light on at 08:00 h] for at least one week prior to the experiments. The surgical procedure was performed according to the methodology previously described by Bennett and Xie [22]. Rats were anesthetized with 10% chloral hydrate (300 mg/kg,

i.p.). The sciatic nerve was exposed by blunt dissection, and four loose ligatures of sterilized 2-0 silk thread were tied around the sciatic nerve. The wound was closed and secured with suture clips. For sham-operated animals, the same surgical procedure was followed, but no ligatures were applied. Rats exhibiting postoperative neurological deficits or poor grooming were excluded from the experiments. All procedures were carried out in accordance with the institutional guidelines for animal care and use. All efforts were made to minimize both the suffering and number of animals used.

1.2 *Behavioral tests*

1.2.1 *Thermal hyperalgesia*

The method of Hargreaves *et al*. [23] was used to assess paw withdrawal latency to a thermal nociceptive stimulus. To assess thermal nociceptive responses, a plantar analgesia instrument (BME2410A, Institute of Biological Medicine, Academy of Medical Science, China) was used. Animals were placed in individual plastic boxes and allowed to adjust to the environment for 30 min. Five measurements of thermal nociceptive threshold were taken for each rat, at 5 min intervals, and the mean of 5 measurements was regarded as thermal withdrawal latency (TWL). A 20 s cutoff was used to prevent tissue damage.

1.2.2 *Mechanical allodynia*

The mechanical allodynia was assessed according to the method of Chaplan *et al*. [24] by Von Frey test. Each rat was placed in a clear plastic box (20 cm × 17 cm × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. Von Frey filaments (Stoelting, Wood Dale, IL, USA), 1.4–15 g bending force, were applied to the midplantar skin of the hind paw with each application holding for 6 s. The mechanical withdrawal threshold (MWT) was measured by using up-down method [25].

1.3 *Drugs administration*

We carried out the drug evaluation on the 7th day after nerve injury, when CCI rats were at peak levels of behavioral sensitization. The behavior tests were performed immediately before (0 h) and at 1, 3, 6, 12 h after administration. Ro 25-6981 and anti-BNDF antibody (Millipore, USA) were dissolved in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4). Rats were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.) and the head fixed in a stereotaxic instrument (Narishige Scientific Instruments, Tokyo, Japan). A 3 μL volume of either drugs was injected into one LV according to stereotaxic coordinates [Bregma: (−1.2 ± 0.4) mm, Depth: (3.2 ± 0.4) mm, Right of median sagittal plane: (1.4 ± 0.2) mm].

1.4 *Double-labeled immunofluorescence staining*

For retrograde labeling of CSF-CN, 3 μL 30% CB-HRP (Sigma, USA) was injected into the LV 48 h before tissue processing. Rats were deeply anesthetized with 10% chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 0.01 mol/L PBS (150 mL, pH 7.4), which was followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB, 300 mL, pH 7.4) for fixation. The brainstem was harvested and post-fixed overnight in the same fixative and then cryoprotected in 30% sucrose in 0.1 mol/L PB until tissue blocks sank to the bottom. Sections were cut on a cryostat (Leica CM1900, Germany) at 40 μm in the transverse plane.

Double-labeled immunofluorescence staining was used to assess if GluN2B or BDNF was expressed in the CSF-CN. Sections were blocked with normal donkey serum for 30 min at room temperature and incubated with primary antibodies overnight at 4 °C. Primary antibodies included goat anti-cholera toxin B-subunit (1∶400, Millipore), rabbit anti-GluN2B (1∶200, Abcam, UK), and rabbit anti-BDNF (1∶200, Millipore). Sections were then incubated for 2 h at room temperature with a corresponding Alexa Fluor 488-conjugated or Alexa Fluor 594-conjugated secondary antibody (1∶200, Invitrogen, USA). Images were obtained using a confocal laser microscope (FV1000; Olympus, Tokyo, Japan).

1.5 *Western blot*

Equal amounts of protein (60 μg) from the CSF-CN of rats was subjected to 8% SDS-PAGE gels and then transferred to a PVDF membrane. The membrane was blocked with 3% bovine serum albumin (BSA) for 2 h at room temperature, and then incubated overnight at 4 °C with rabbit anti-GluN2B (1∶1 000), rabbit anti-BDNF (1∶1 000), and anti-β-tubulin (1∶1 000, Sigma, USA) as a loading control. The membrane was incubated in the appropriate alkali phosphatase-conjugated goat anti-rabbit IgG (1∶1 000, Sigma) for 2 h at room temperature. Then, the protein band was visualized by the BCIP/NBT Alkaline Phosphatase Color Development Kit. Developed films were digitized using an Epson Perfection 2480 scanner (Seiko Corp., Nagano, Japan). Optical densities of detected proteins were obtained using ImageJ.

1.6 *Statistical analysis*

Values were expressed as mean ± SEM. All statistical

tests were undertaken in SPSS v.16.0. Results were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test to determine differences among the groups. Values of $P < 0.05$ or $P < 0.01$ were considered statistically significant.

2 RESULTS

2.1 Distribution of GluN2B in the CSF-CN of normal rats

To label dCSF-CNs, we applied the fluorescent tracer CB-HRP to the LV in adult rats. Consistent with previ-

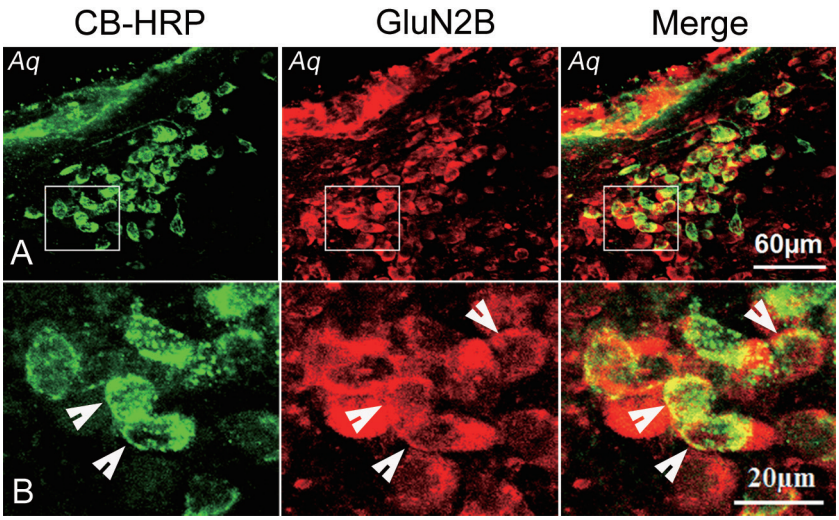

Fig. 1. Distribution of GluN2B in the CSF-CN in the normal SD rats. *A*: Low-magnification confocal images showing single labeling of CB-HRP and GluN2B, and double labeling of these two molecules in the CSF-CN. Aq, aqueduct. Scale bar, 60 μm. *B*: High-magnification confocal images of CSF-CN area. Arrows indicate double labeled neurons. Scale bar, 20 μm.

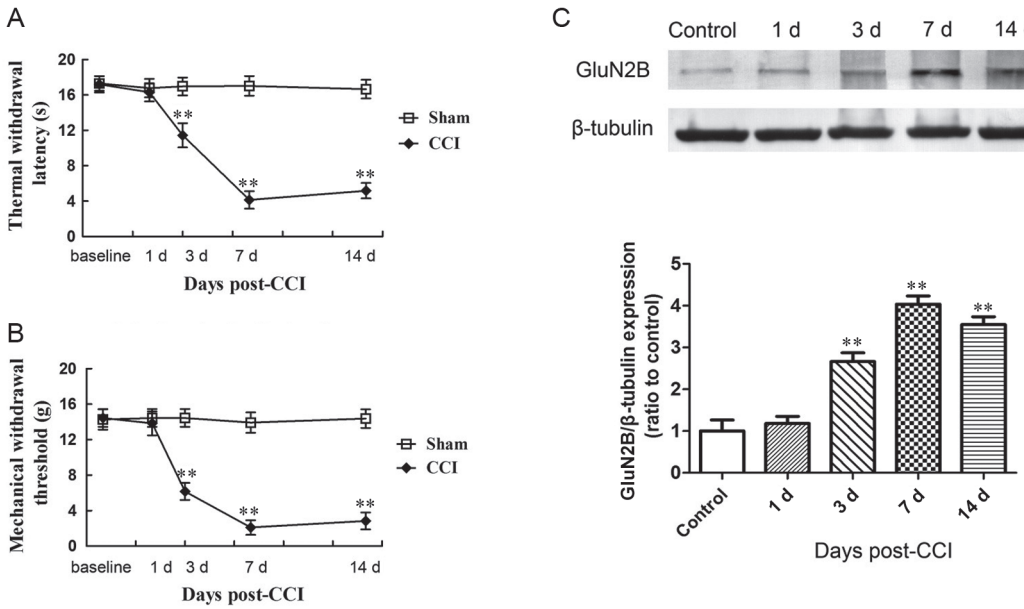

Fig. 2. Assessment of behavioral tests and alteration of GluN2B protein expression in the CSF-CN in CCI rats. *A* and *B*: Time course of values for thermal withdrawal latency (TWL) measured by hot plate test and mechanical withdrawal threshold (MWT) measured by Von Frey filament assay. The CCI-operated rats developed significant thermal hyperalgesia and mechanical allodynia from day 3 to day 14. $^{**}P < 0.01$ compared with sham-operated rats ($n = 6$). *C*: Protein levels of GluN2B at different time points after CCI operation. Control indicates the values of normal rats. $^{**}P < 0.01$ compared with control ($n = 6$). Mean ± SEM.

ous reports, we found that the majority of CB-HRP labeled neurons (green) were localized in the ventral PAG of the brainstem, which formed CSF-CN. To examine whether dCSF-CNs express GluN2B, we used double-labeled immunofluorescence technique. We observed that GluN2B immunoreactive neurons (red) were distributed near the midline of the ventral mesencephalic aqueduct (Aq) and the majority of them were found in CSF-CN. The double-labeled neurons of GluN2B/CB-HRP (yellow) indicated that GluN2B existed in CSF-CN (Fig. 1).

2.2 Alteration of GluN2B protein expression in the CSF-CN following CCI

To investigate whether the alteration of GluN2B level in CSF-CN is related to the mechanism of neuropathic pain, we firstly explored the behavioral performance of hyperalgesia after CCI. Thermal hyperalgesia was measured using the hot plate test. Compared with baseline, the values for TWL of CCI rats decreased significantly from day 3, and reached the minimum on day 7, and remained decreased on day 14 following ligation (Fig. 2*A*). Mechanical allodynia was assessed by Von Frey filament assay. Time course of CCI-induced mechanical allodynia was the same as that of CCI-induced thermal hyperalgesia. Values for MWT of CCI rats began to decrease on day 3, and reached a minimum on day 7, and remained decreased on day 14 post-surgery (Fig. 2*B*). It can be seen that thermal hyperalgesia and mechanical allodynia began on day 3 following CCI and maintained at the peak from day 7 to day 14 after ligature placement.

Injury-induced alteration of GluN2B level in the CSF-CN could underlie the development of behavioral sensitivity. Therefore, we assessed the alteration of gross expression of GluN2B protein in the CSF-CN by Western blot analysis on day 1, 3, 7, and 14 after CCI. Analysis revealed that expression of GluN2B protein level increased notably on day 3, and reached the peak on day 7, and remained elevated on day 14 after nerve injury (Fig. 2*C*).

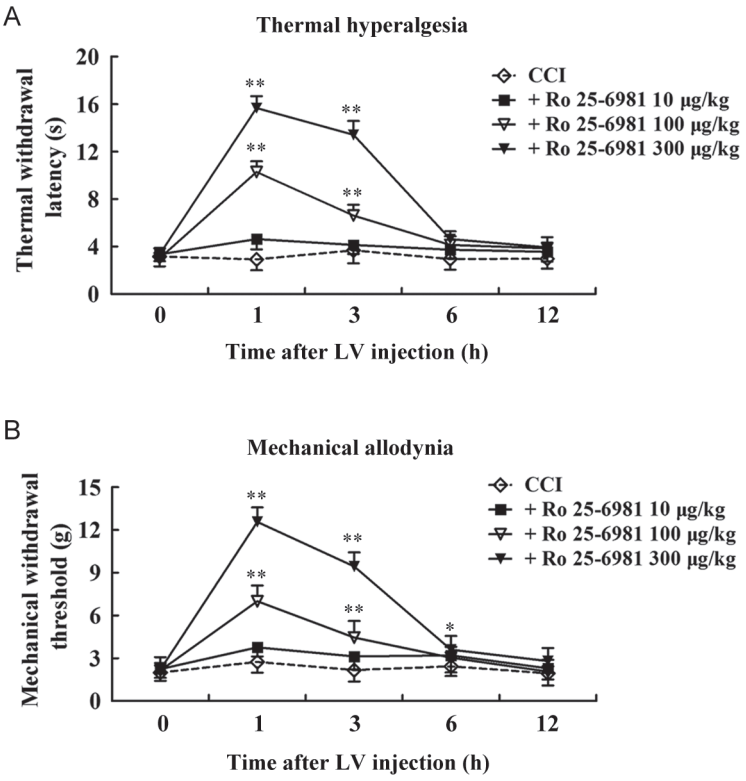

Fig. 3. Effects of Ro 25-6981 (a GluN2B antagonist) on CCI-induced thermal hyperalgesia and mechanical allodynia. Ro 25-6981 (10–300 μg/kg) was injected on day 7 after the CCI operation. Intracerebroventricular (ICV) injection of Ro 25-6981 dose-dependently increased the values for thermal withdrawal latency (TWL, *A*) in response to noxious thermal stimulus and values for mechanical withdrawal threshold (MWT, *B*) in response to noxious mechanical stimulus in CCI rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with CCI group injected with PBS ($n = 6$). Mean ± SEM.

2.3 Selective GluN2B antagonist attenuated thermal hyperalgesia and mechanical allodynia

Our results showed that GluN2B expression was increased in the CSF-CN after nerve injury, suggesting that inhibition of GluN2B subunit in the CSF-CN may alter CCI-induced behavioral responses. To test this hypothesis, we injected a selective GluN2B antagonist, Ro 25-6981 into the LV of CCI rats on the 7th day after the establishment of pain model, when CCI rats were at peak levels of behavioral sensitization. The behavioral tests revealed that intracerebroventricular (ICV) injection of 300 μg/kg or 100 μg/kg, but not 10 μg/kg, Ro 25-6981 significantly increased the values for TWL in response to noxious thermal stimulus and values for MWT in response to noxious mechanical stimulus in CCI rats. ICV administration of Ro 25-6981 (100–300 μg/kg) significantly inhibited the reduction of TWL and MWT after CCI at 1 and 3 h after administration. The effects of Ro 25-6981 disappeared by 12 h after administration (Fig. 3).

2.4 Blocking BDNF signaling suppressed nerve injury-induced neuropathic pain

Previous studies indicated that increased expression and secretion of BDNF had been implicated in injury-induced neuropathic pain in the sensory system [26]. To determine whether BDNF signaling in the CSF-CN is required for development and maintenance of neuropathic pain, we tested the dose-response effects of ICV injection of a neutralizing antibody against BDNF on day 7 after CCI. Acute administration of the antibody (10–50 μg, ICV) completely reversed the reduction of PWL and MWT induced by CCI at 1 and 3 h after administration. The effects of anti-BDNF antibody disappeared by 12 h after administration (Fig. 4).

2.5 Changes of BDNF signaling in the CSF-CN of CCI rats

We have found that the BDNF signaling was involved in the development of the hyperalgesia induced by CCI. In order to further elucidate the role of BDNF signaling

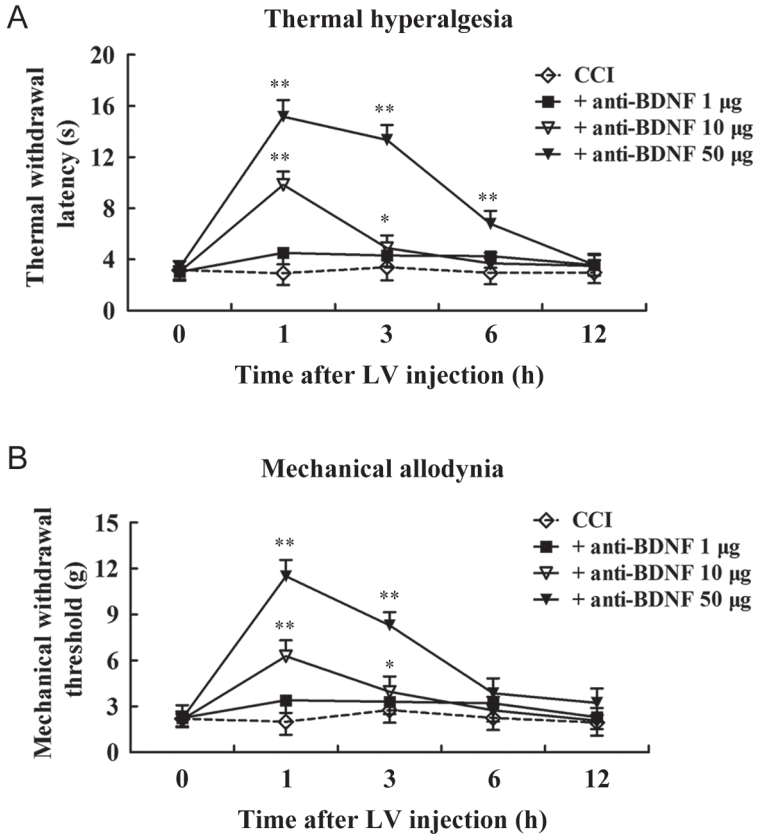

Fig. 4. Effects of a neutralizing antibody against BDNF on CCI-induced thermal hyperalgesia and mechanical allodynia. Anti-BDNF antibody (1–50 μg) was injected on day 7 after the CCI operation. ICV injection of anti-BDNF antibody dose-dependently increased the values for thermal withdrawal latency (TWL, *A*) in response to noxious thermal stimulus and values for mechanical withdrawal threshold (MWT, *B*) in response to noxious mechanical stimulus in CCI rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with CCI group injected with PBS ($n = 6$). Mean ± SEM.

in the CSF-CN in the mechanism of chronic neuropathic pain, we carried out the double-labeled immunofluorescence technique and Western blot analysis. The double-labeled immunofluorescence staining revealed that BDNF immunoreactive neurons distributed predominantly in the CSF-CN (Fig. 5*A*, *B*). The Western blot analysis suggested that the expression of BDNF protein was increased significantly on day 1 and remained elevated 14 days after nerve injury (Fig. 5*C*).

2.6 Effects of the selective GluN2B antagonist on regulation of BDNF signaling in CCI rats

Considering Ro 25-6981 could greatly change the behavioral sensitization of CCI rats, whether the influence of the selective GluN2B antagonist on neuropathic reflex sensitization was relevant to the alteration in BDNF level was our next concern. To determine the role of inhibition of GluN2B in regulation of BDNF signaling, Western blot analysis was used to examine the expression of BDNF protein in CSF-CN at 1 h after ICV application of Ro 25-6981 (300 μg/kg) on day 7 following CCI. We observed that the expression of BDNF protein in CSF-CN increased significantly on day 7 after CCI and the increased expression was reversed by ICV injection of Ro 25-6981 (Fig. 6).

3 DISCUSSION

In this study, we used CB-HRP, a fluorescent tracer which did not pass through the spaces of the ependyma

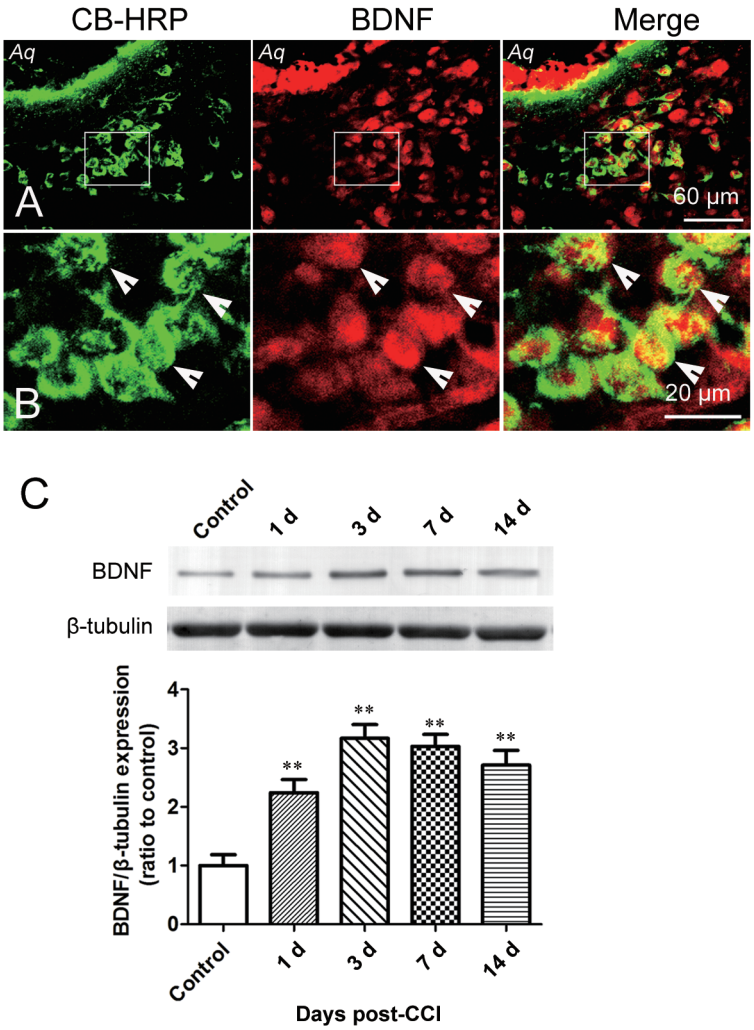

Fig. 5. Changes of BDNF signaling in the CSF-CN in CCI rats. *A*: Low-magnification confocal images showing single labeling of CB-HRP and BDNF, and double labeling of these two molecules in the CSF-CN. Aq, aqueduct. Scale bar, 60 μm. *B*: High-magnification confocal images of CSF-CN area. Arrows indicating double labeled neurons. Scale bar, 20 μm. *C*: Protein levels of BDNF at different time points after CCI operation. Control indicates the values of normal rats. $^{**}P < 0.01$ compared with control ($n = 6$). Mean ± SEM.

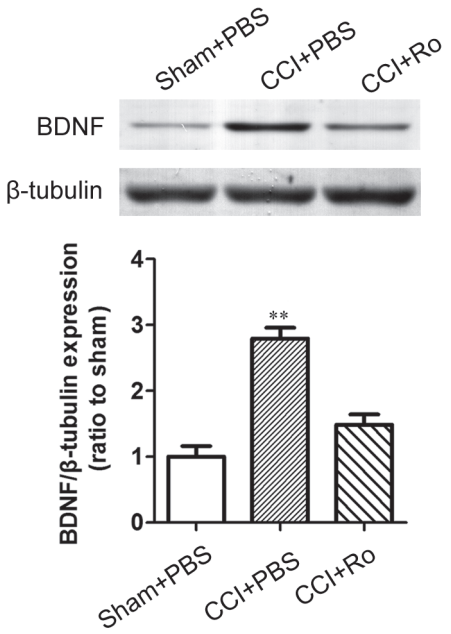

Fig. 6. The effects of the selective GluN2B antagonist on regulation of BDNF signaling in CCI rats. Western blot analysis indicated that ICV injection of Ro 25-6981 (Ro, 300 μg/kg) significantly reduced the expression of BDNF in the CSF-CN on day 7 after CCI operation. $^{**}P < 0.01$ compared with sham+PBS ($n = 6$). Mean ± SEM.

and diffused into the parenchyma when injected into the ventricle of the brain, to retrogradely label CSF-CN in adult rats. This approach allowed us to discern the protein expression profiles of CSF-CN. The results showed that the CSF-CN was predominantly localized in the ventral PAG of the mesencephalon, both GluN2B and BDNF were distributed near the Aq, and the majority of them were found in the CSF-CN. Moreover, we found CCI rats exhibited increased levels of GluN2B and BDNF protein in the CSF-CN and both selective GluN2B antagonist Ro 25-6981and anti-BDNF antibody completely reversed the CCI-induced thermal hyperalgesia and mechanical allodynia when administered after the development of neuropathic pain. These findings provide the convergent evidence that the peripheral nerve injury induces up-regulation of GluN2B and BDNF protein expression in the CSF-CN and that increased expression therein is essential for the development and maintenance of nerve injury-induced neuropathic pain.

Considering the special structure properties of CSF-CN and its locations within ventral PAG, which constitutes a pain-control system that descends from the brain onto the spinal cord, it raises the possibility that CSF-CN may participate in injury-induced hyperalgesia through nonsynaptic signal transmission via releasing or absorbing bioactive substances. Our previous studies indicated that regulation of substance P, drebrin, and 5-HT1A expression contributed to nerve injury-induced inflammatory and neuropathic pain [4, 27, 28]. There is emerging evidence that NMDARs, especially GluN2B-containing NMDAR, are thought to play important roles in the processes of central sensitization and pathogenesis of neuropathic pain [13]. However, little is known about the roles of GluN2B in the CSF-CN and its downstream targets in processing the nerve injury-induced neuropathic pain.

NMDARs undergo plastic changes in physiological or pathological conditions [10, 29]. The changes in NMDAR subunit composition also have consequences for activity dependent plasticity [14]. Previous research has shown that transgenic overexpression of GluN2B subunits in the PAG increases behavioral responses to persistent inflammatory pain [30]. Selective inhibition of GluN2B in spinal dorsal horn effectively alleviated spinal cord injury-induced neuropathic pain, suggesting the up-regulation of GluN2B function was involved in central sensitization [13]. In the present study, we demonstrated that changes of GluN2B expression in the CSF-CN was sufficient for the development of injury-induced thermal hyperalgesia and mechanical allodynia.

To investigate whether BDNF signaling in the CSF-CN contributes to the incidence of thermal hyperalgesia and mechanical allodynia, we examined the effects of anti-BDNF antibody on the CCI-induced pain behaviors. ICV injection of the anti-BDNF antibody inhibited the pain behaviors, suggesting that BDNF signaling is involved in the nerve injury-induced thermal hyperalgesia and mechanical allodynia. This is further supported by the finding that the expression of BDNF protein in the rat CSF-CN was increased after CCI, and this increased expression was reversed by ICV injection of Ro 25-6981, which attenuated the CCI-induced pain behavior. Recent experiments revealed that the BDNF signaling played an important role in the processes of central sensitization and pathogenesis of neuropathic pain [20, 31]. In the CCI model, the sciatic nerve transection model, the spinal nerve ligation model and the spinal nerve transection model of neuropathic pain, increases in BDNF signaling activity were visualized in the superficial dorsal horn [32]. Taken together, these findings suggest that BDNF signaling in CSF-CN contributes to the incidence of CCI-induced thermal hyperalgesia and

mechanical allodynia.

Interestingly, the present study results showed that both CCI-induced pain behaviors and increase of BDNF signaling were reversed by ICV injection of Ro 25-6981, a selective GluN2B antagonist. Previous studies also revealed that BDNF signaling pathway in the spinal cord contributed to the development and maintenance of nerve injury-induced neuropathic pain by activation of the dorsal horn GluN2B-containing NMDAR [21]. Thus, the GluN2B-containing NMDAR and BDNF signaling comprise a local circuit that amplifies the signal of pain transmission. If sustained production of these factors after CCI is required for maintenance of central sensitization, blockade of this circuit by the GluN2B antagonist would likely reduce pain excitatory neurotransmission in the CSF-CN. In present study, we demonstrated that inhibition of GluN2B in the CSF-CN not only alleviated nerve injury-induced thermal hyperalesia and mechanical allodynia, but also reversed the increased expression of BDNF signaling induced by CCI. All of these findings indicate that GluN2B antagonists have analgesic effects on the CCI-induced hyperalgesia through regulation of BDNF signaling at the CSF-CN level.

Taken together, our findings provide strong evidence that up-regulation of GluN2B-containing NMDAR in the CSF-CN is involved in the modulation of the nerve injury-induced neuropathic pain. Targeting GluN2B-BDNF pathway in the CSF-CN may be a useful alternative or adjunct therapy for CCI-induced peripheral neuropathy.

REFERENCES

1 Zhang LC, Zeng YM, Ting J, Cao JP, Wang MS. The distributions and signaling directions of the cerebrospinal fluid contacting neurons in the parenchyma of a rat brain. Brain Res 2003; 989(1): 1–8.

2 Lu X, Geng X, Zhang L, Zeng Y. The methodology for labeling the distal cerebrospinal fluid-contacting neurons in rats. J Neurosci Methods 2008; 168(1): 98–103.

3 Jiang WX (蒋文旭), Zhang LC. Distribution and effects of 5-HT_{1A} receptors in distal cerebral spinal fluid-contacting neurons in rat brain parenchyma in neuropathic pain. Acta Physiol Sin (生理学报) 2008; 60(2): 243–248 (in Chinese with English abstract).

4 Liu PF, Fang HZ, Yang Y, Zhang QQ, Zhou QQ, Chen SS, Zhou F, Zhang LC. Activation of P2X3 receptors in the cerebrospinal fluid-contacting nucleus neurons reduces formalin-induced pain behavior via PAG in a rat model. Neuroscience 2017; 358: 93–102.

5 Chen J, Li L, Chen SR, Chen H, Xie JD, Sirrieh RE, MacLean DM, Zhang Y, Zhou MH, Jayaraman V, Pan HL. The alpha2delta-1-NMDA receptor complex is critically involved in neuropathic pain development and gabapentin therapeutic actions. Cell Rep 2018; 22(9): 2307–2321.

6 Deng M, Chen SR, Pan HL. Presynaptic NMDA receptors control nociceptive transmission at the spinal cord level in neuropathic pain. Cell Mol Life Sci 2019; 76(10): 1889–1899.

7 Lefevre Y, Amadio A, Vincent P, Descheemaeker A, Oliet SH, Dallel R, Voisin DL. Neuropathic pain depends upon *D*-serine co-activation of spinal NMDA receptors in rats. Neurosci Lett 2015; 603: 42–47.

8 Renno WM. Prolonged noxious stimulation increases periaqueductal gray NMDA mRNA expression: a hybridization study using two different rat models for nociception. Ir J Med Sci 1998; 167(3): 181–192.

9 Zanos P, Nelson ME, Highland JN, Krimmel SR, Georgiou P, Gould TD, Thompson SM. A negative allosteric modulator for alpha5 subunit-containing GABA receptors exerts a rapid and persistent antidepressant-like action without the side effects of the NMDA receptor antagonist ketamine in mice. eNeuro 2017; 4(1): ENEURO.0285-16.2017. doi: 10.1523/ENEURO.0285-16.2017.

10 Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 1994; 12(3): 529–540.

11 Gong WY, Wang R, Liu Y, Jin H, Zhao ZW, Wang YL, Li HY, Zhang X, Ni JX. Chronic monoarthritis pain accelerates the processes of cognitive impairment and increases the NMDAR subunits NR2B in CA3 of hippocampus from 5-month-old transgenic APP/PS1 mice. Front Aging Neurosci 2017; 9: 123.

12 Xia T, Cui Y, Qian Y, Chu S, Song J, Gu X, Ma Z. Regulation of the NR2B-CREB-CRTC1 signaling pathway contributes to circadian pain in murine model of chronic constriction injury. Anesth Analg 2016; 122(2): 542–552.

13 Kim Y, Cho HY, Ahn YJ, Kim J, Yoon YW. Effect of NMDA NR2B antagonist on neuropathic pain in two spinal cord injury models. Pain 2012; 153(5): 1022–1029.

14 Zhuo M. Plasticity of NMDA receptor NR2B subunit in memory and chronic pain. Mol Brain 2009; 2: 4.

15 Chizh BA, Reissmuller E, Schlutz H, Scheede M, Haase G, Englberger W. Supraspinal vs spinal sites of the antinociceptive action of the subtype-selective NMDA antagonist ifenprodil. Neuropharmacology 2001; 40(2): 212–220.

16 M'Dahoma S, Barthelemy S, Tromilin C, Jeanson T, Viguier F, Michot B, Pezet S, Hamon M, Bourgoin S. Respective pharmacological features of neuropathic-like pain evoked by intrathecal BDNF versus sciatic nerve ligation in rats. Eur

Neuropsychopharmacol 2015; 25(11): 2118–2130.
17 Zhang L, Wang G, Ma J, Liu C, Liu X, Zhan Y, Zhang M. Brain-derived neurotrophic factor (BDNF) in the rostral anterior cingulate cortex (rACC) contributes to neuropathic spontaneous pain-related aversion via NR2B receptors. Brain Res Bull 2016; 127: 56–65.
18 Zhou J, Wang F, Xu C, Zhou Z, Zhang W. The histone demethylase JMJD2A regulates the expression of BDNF and mediates neuropathic pain in mice. Exp Cell Res 2017; 361(1): 155–162.
19 Chen HS, Zhou ZH, Li M, Wang JX, Liu BJ, Lu Y. Contribution of brain-derived neurotrophic factor to mechanical hyperalgesia induced by ventral root transection in rats. Neuroreport 2013; 24(4): 167–170.
20 Retamal J, Reyes A, Ramirez P, Bravo D, Hernandez A, Pelissier T, Villanueva L, Constandil L. Burst-like subcutaneous electrical stimulation induces BDNF-mediated, cyclotraxin B-sensitive central sensitization in rat spinal cord. Front Pharmacol 2018; 9: 1143.
21 Geng SJ, Liao FF, Dang WH, Ding X, Liu XD, Cai J, Han JS, Wan Y, Xing GG. 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 2010; 222(2): 256–266.
22 Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988; 33(1): 87–107.
23 Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988; 32(1): 77–88.
24 Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994; 53(1): 55–63.
25 Dixon WJ. Efficient analysis of experimental observations. Annu Rev Pharmacol Toxicol 1980; 20: 441–462.
26 Sun W, Zhang L, Li R. Overexpression of miR-206 ameliorates chronic constriction injury-induced neuropathic pain in rats via the MEK/ERK pathway by targeting brain-derived neurotrophic factor. Neurosci Lett 2017; 646: 68–74.
27 Avila-Rojas SH, Velazquez-Lagunas I, Salinas-Abarca AB, Barragan-Iglesias P, Pineda-Farias JB, Granados-Soto V. Role of spinal 5-HT5A, and 5-HT1A/1B/1D, receptors in neuropathic pain induced by spinal nerve ligation in rats. Brain Res 2015; 1622: 377–385.
28 Geng XJ (耿晓娟), Lu XF, Zhang LC, Zeng YM. Expression of drebrin in the distal cerebrospinal fluid contacting neurons of rats with chronic constriction injury of sciatic nerve. Acta Physiol Sin (生理学报) 2008; 60(4): 469–474 (in Chinese with English abstract).
29 Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 1994; 368(6467): 144–147.
30 Hu J, Wang Z, Guo YY, Zhang XN, Xu ZH, Liu SB, Guo HJ, Yang Q, Zhang FX, Sun XL, Zhao MG. A role of periaqueductal grey NR2B-containing NMDA receptor in mediating persistent inflammatory pain. Mol Pain 2009; 5: 71.
31 Uchida H, Matsushita Y, Ueda H. Epigenetic regulation of BDNF expression in the primary sensory neurons after peripheral nerve injury: implications in the development of neuropathic pain. Neuroscience 2013; 240: 147–154.
32 Vanelderen P, Rouwette T, Kozicz T, Roubos E, Van Zundert J, Heylen R, Vissers K. The role of brain-derived neurotrophic factor in different animal models of neuropathic pain. Eur J Pain 2010; 14(5): 473.e1–e9.