

## Basic Science

## Extracellular Signal-Regulated Kinase 5 in the Cerebrospinal Fluid-Contacting Nucleus Contributes to Neuropathic Pain in Rats

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**Background:** The activation of mitogen-activated protein kinases (MAPKs) have been observed in synaptic plasticity processes of learning and memory in neuropathic pain. Cerebrospinal fluid-contacting nucleus (CSF-CN) has been identified with the onset and persistence of neuropathic pain. However, whether extracellular signal-regulated protein kinase 5 (ERK5), a member of MAPKs, in CSF-CN participates in neuropathic pain has not been studied yet.

**Objective:** The aim of the present study was to identify the role of ERK5 in CSF-CN on the formation and development of neuropathic pain, and to investigate its possible mechanism.

**Study Design:** Controlled animal study.

**Setting:** University laboratory.

**Methods:** After a chronic constriction injury (CCI) model was produced, BIX02188 was dissolved in 1% DMSO and injected into the lateral ventricles LV in a volume of 3  $\mu$ l with different doses (0.1  $\mu$ g, 1  $\mu$ g, 10  $\mu$ g). Mechanical allodynia and thermal hypersensitivity behavioral test, immunofluorescence, and western blot technique were used in this research.

**Result:** Following CCI, mechanical allodynia and thermal hypersensitivity were developed within a day, peaked at 14 days, and persisted for 21 days. ERK5 was remarkably activated by CCI in CSF-CN. Moreover, selective inhibiting of p-ERK5 expression in CSF-CN by BIX02188 could significantly relieve CCI-induced mechanical allodynia and thermal hypersensitivity, accompanying with the decreased phosphorylation of cAMP response-element binding protein (CREB) in CSF-CN.

**Limitations:** More underlying mechanism(s) of the role of ERK5 in CSF-CN on the formation and development of neuropathic pain will be needed to explore in future research.

**Conclusion:** These findings suggest activation of ERK5 in CSF-CN might contribute to the onset and development of neuropathic pain and its role might be partly accomplished by p-CREB.

**Key words:** Neuropathic pain, extracellular signal-regulated kinase 5, distal cerebrospinal fluid-contacting neurons, cerebrospinal fluid-contacting nucleus, chronic constriction injury, cAMP response-element binding protein

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**N**europathic pain, defined as pain caused by an injury of the somatosensory system at the peripheral and central level, is characterized by allodynia, thermal hyperalgesia, and spontaneous

pain (1-2). To date, despite considerable progress, the molecular and cellular mechanisms underlying neuropathic pain are not yet completely understood, and, thus, the treatment for it remains a challenge.

Extracellular signal-regulated protein kinase 5 (ERK5), a member of mitogen-activated protein kinases (MAPKs) family, contains a Thr-Glu-Tyr dual phosphorylation motif of the ERK1/2-type MAP kinase. However, it has a unique loop-12 structure and an unusually large C-terminal non-kinase domain, making ERK5 quite different from ERK1/2 (3). Accumulative evidence indicates that ERK5 is involved in neuronal plasticity, including learning and memory, drug addiction, and pain hypersensitivity (4-8). Activation of ERK5 in rat spinal dorsal horn and dorsal root ganglion (DRG) remarkably increases by nerve injury, and antisense knockdown of ERK5 suppresses nerve injury-induced hyperalgesia (5). Moreover, inflammatory pain induced by injection of complete Freund's adjuvant into rat hind paw produces notable activation of ERK5 in dorsal root ganglion and spinal dorsal horn neurons (4,8). All of these results have indicated that ERK5 might play an important role in the development and maintenance of pathological pain.

The cerebrospinal fluid-contacting neurons are a peculiar type of neurons in the central nervous system. According to the cytologic positions of the neurons, they can be divided into 3 types: an intraependyma neuron which lines the walls of ventricles and the central canal of the spinal cord, a supraependyma cell which is subjacent to the ependymal, and distal cerebrospinal fluid-contacting neurons (dCSF-CNs) whose bodies are in the parenchyma of the brain and whose processes extend into the CSF in the cavity of the ventricle in the central nervous system (9-10). Horseradish peroxidase-conjugated toxin subunit B (CB-HRP), which could be absorbed by dCSF-CNs but not pass through the tight juncture of the ependyma, was used to be a tracer to understand the function of dCSF-CNs (10). Through this approach and morphological methods, we found that dCSF-CNs were a distinct group from other neurons, whose bodies are mainly located in the ventral periaqueductal central gray (PAG) of the brainstem and whose processes penetrate the ependymal cells and stretch into the CSF. In view of the anatomical and morphological characteristics, we named them cerebrospinal fluid-contacting nucleus (CSF-CN) (6,11). For decades, substantial evidence has established that multiple sites are closely related to neuropathic pain, including the somatosensory cortex, dorsal thalamus, rostral anterior cingulate cortex (rACC), amygdale nucleus, prefrontal cortex (PFC), and spinal cord (12-17). However, our recent research revealed that CSF-CN, as a connection between the parenchyma of the brain and the CSF sys-

tems, might play an important role in neuropathic pain (18-20). Accordingly, the aim of the present study was to determine whether the activation of ERK5 in CSF-CN contributes to neuropathic pain.

## **METHODS**

### **Subjects**

All experiment protocols were approved by the Animal Care and Use Committee of Xuzhou Medical College and were in accordance with the ethical guidelines of the International Association for the Study of Pain (21). The animals were provided by Experimental Animal Center of Xuzhou Medical College. Male Sprague-Dawley rats (220 – 250 g) were housed on a 12/12 h light/dark cycle, with free access to food and water.

### **Chronic constriction injury model**

The chronic constriction injury (CCI) model was produced following the method of Bennett and Xie (22). Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal) and the right sciatic nerve was exposed by the blunt preparation of connective tissue at the mid-thigh level, proximal to the sciatic trifurcation. Four sterile 4-0 chromic gut ligatures were loosely tied so as to barely constrict blood flow. In sham-operated animals, the sciatic nerve was exposed but not ligated. The skin was sutured and the anesthesia discontinued. The rats were transferred to a recovery cage until they showed signs of locomotive competence and a reproducible righting reflex.

### **Measurement of Thermal Hyperalgesia**

The II TC Plantar Analgesia Meter (IITC Life Science Inc., CA) was used to measure paw withdrawal latency, as described by Hargreaves et al (23). Rats were placed in plastic chambers (7×9×11 cm) to adapt to the environment for one hour before testing. The radiant heat was directed to the plantar surface of each hind paw that was flushed against the glass. The nociceptive endpoints in the radiant heat test were the characteristic lifting or licking of the hind paw. The time to the endpoint was considered the paw-withdrawal latency (PWL). An automatic 25 second cutoff was used to prevent tissue damage. Thermal stimuli were delivered 3 times to each hind paw at 5-minute intervals.

### **Measurement of Mechanical Allodynia**

Mechanical allodynia was assessed with electronic

Von Frey filaments (II TC Life Science Inc., CA). Rats were placed in individual plastic boxes (20×25×15 cm) on a metal mesh floor and allowed to acclimate for one hour before testing. The filaments were presented perpendicularly to the plantar surface, and brisk withdrawal or paw flinching were considered as positive responses, the digital number presented on the monitor was recorded as the paw withdrawal threshold (PWT).

### Drug Application

Rats were anesthetized with sevoflurane and the head fixed in a stereotaxic instrument. Forty-eight hours before they were perfused for immunofluorescence, a 3- $\mu$ l volume of 30% CB-HRP (Sigma) was injected into one of the rat's lateral ventricles (LV) according to stereotaxic coordinates (Bregma:  $-1.2 \pm 0.4$  mm, Depth:  $3.2 \pm 0.4$  mm, Right of median sagittal plane:  $1.4 \pm 0.2$  mm). BIX02188, the latest selective inhibitor of ERK5, was purchased from Boehringer Ingelheim (Ridgefield, CT), which was dissolved in 1% DMSO and injected into the LV in a volume of 3  $\mu$ l with different doses (0.1  $\mu$ g, 1  $\mu$ g, 10  $\mu$ g), the control for BIX02188 administration was 1% DMSO.

### Immunofluorescence

Forty-eight hours following CB-HRP injection, rats were deeply anesthetized with sevoflurane and perfused with 150 mL of phosphate buffered saline (PBS) (0.01 M PBS, pH 7.4), followed without interruption by 4% paraformaldehyde in 0.2 M phosphate buffer (300 ml, pH 7.4). The brainstem was removed immediately and post-fixed for 4 – 6 hours at 4°C, then cryoprotected by immersion for 24 – 48 hours in sucrose gradients (5%, 10%, 15%, 20%, and 30%) with 0.01 mol/L PBS at 4°C. The brainstem embedded with OCT at -20°C and sectioned on a cryostat (Leica CM1900, Germany) at 40  $\mu$ m in the transverse plane.

In order to determine the expression of p-ERK5 in CSF-CN, the frozen sections were collected in PBS. Following 3 washes in PBS, sections were incubated in PBS with 0.3% triton X-100 for 48 – 72 hours at 4°C with a goat anti-cholera toxin B-subunit (1:200, Sigma) and in rabbit anti-p-ERK5 (1:500, Thermo). After rinsing in PBS, sections were incubated in donkey anti-goat IgG conjugated to Texas Red (1:400, Abcam), and in donkey anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (1:400, Abcam) in the dark for 2 hours at room temperature. Finally, sections were rinsed, mounted, and cover slipped with glycerol containing 2.5% of anti-fading agent DABCO (1, 4-di-aza-bi-cyclo-2, 2, 2-octane, Sigma) and stored at -20°C in the dark. Tissue sections

were examined using laser scanning confocal microscopy (Leica, Germany) to identify dCSF-CNs labeled with TR and p-ERK5 labeled with FITC.

### Western Blot

Rats were sacrificed and the CSF-CN samples were quickly extracted under deep isoflurane anesthesia and stored in liquid nitrogen. Tissue samples were homogenized in 50 mM RIPA buffer (20  $\mu$ l/mg), containing protease inhibitor cocktail and phosphatase inhibitor cocktail. Lysates were centrifuged at 14,400 g for 60 minutes at 4 °C, and the concentration of protein in each sample (supernatant) was determined by the Lowry method. Samples with equal amounts of protein were then separated by 10 – 20% PAGE, and the resolved proteins were electro-transferred to nitrocellulose membrane. Membranes were incubated with 5% nonfat milk with for at least 60 minutes at room temperature and incubated with the following primary antibodies: p-ERK5, ERK5 (1:1000; Cell Signaling Technology) and p-CREB, CREB (1:500; SAB) at 4 °C overnight. The membranes were extensively washed with Tris Buffered Saline Tween (TBST) and incubated for 2 hours with the secondary antibody conjugated with alkaline phosphatase (1:1000) at room temperature. The immune complexes were detected by using a NBT/BCIP assay kit (Sigma). Western blot densitometry analysis of signal intensity was performed using Adobe Photoshop software (Adobe, San Jose, CA), and phosphorylation levels of ERK5 from densitometry were normalized to total ERK5. The blot density from control groups was set as 100%.

### Statistical Analyses

For statistical analysis, GraphPad Prism 5 (GraphPad Software, USA) was used. Data were expressed as mean  $\pm$  SEM. Statistical comparison of more than 2 groups was performed using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. The significance of any differences in behavior test was assessed using 2-way ANOVA (within-subject factor:time; between-subjects factor:treatment). A value of  $P < 0.05$  was considered as statistically significant.

## RESULTS

### Chronic constriction injury induced nociceptive behavioral and activation of ERK5

CCI, a well-characterized nerve injury model, was used to discuss neuropathic pain in this study. The pain

threshold of PWT and PWL decreased after CCI surgery and reached the lowest point at 14 days post-surgery ( $*P < 0.05$  at 1 day,  $**P < 0.01$  from 3 to 21 days, CCI group vs sham group; Fig. 1). Thus, 14 days post-operation, as a critical time point, was chosen for drug application and the collection of CSF-CN samples in this research. However, the sham group rats did not exhibit mechanical allodynia and thermal hyperalgesia. In the present study, ERK5 activation was evaluated by detecting the expression of p-ERK5 via immune-fluorescence analysis. The results revealed that the expression of p-ERK5 in CSF-CN increased in the CCI group at 14 days post-CCI compared with the sham operation rats ( $**P < 0.01$ , compared with sham group; Fig. 2).

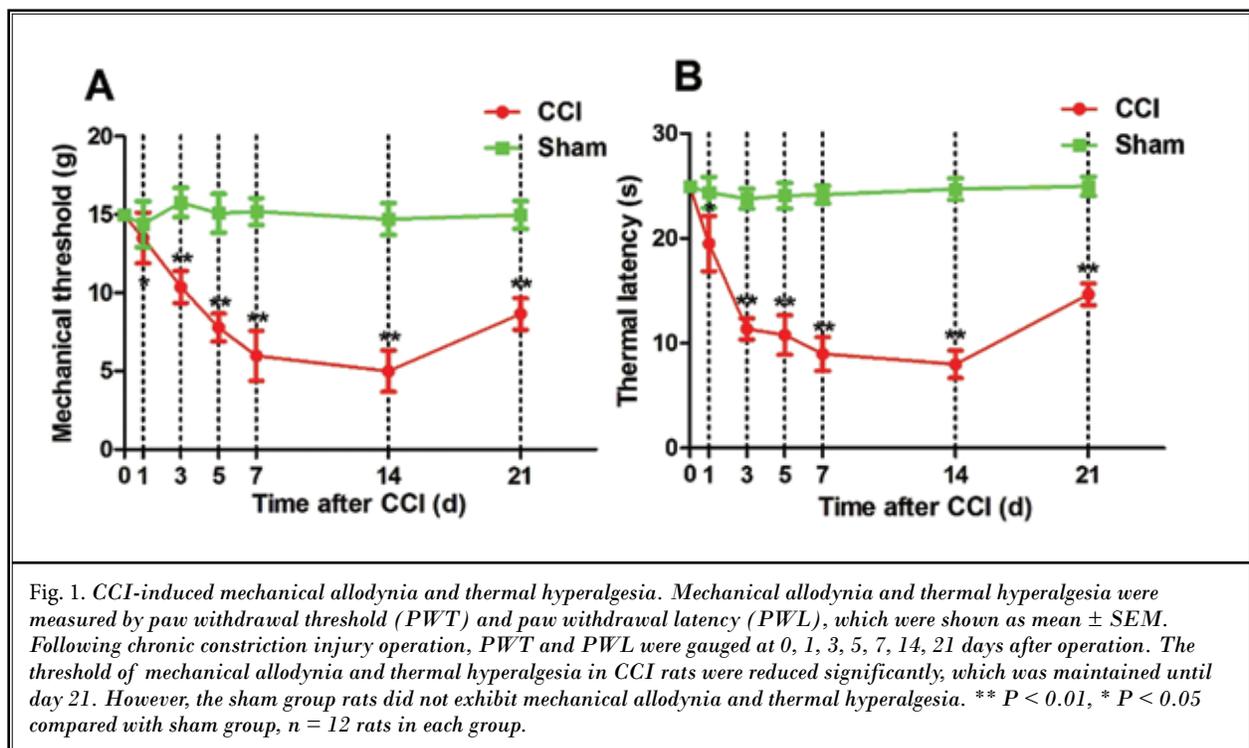
Intra-lateral ventricles injection BIX02188 attenuated chronic constrictive injury-induced mechanical allodynia and thermal hypersensitivity.

To clarify the analgesic property of BIX02188, it was intra-lateral ventricles injected to CCI rats at surgery day 14. The improvement of mechanical allodynia and thermal hypersensitivity was measured at 30 minutes after intra-lateral ventricles injection of BIX02188, and the most significant reduction was detected at one hour after intra-lateral ventricles injection of BIX02188. Thus, one hour after intra-lateral ventricles injection, as

a critical time point, was used for behavior test in this study. The relief was maintained at least up to 4 hours and returned to baseline levels at 8 hours after intra-lateral ventricles injection of BIX02188 ( $**P < 0.01$  at 0.5 h, 1 h, 2 h,  $*P < 0.05$  at 4 h, vs. 0 h; Fig. 3A). To further determine the pre-treatment effect of BIX02188 on the improvement of mechanical allodynia and thermal hypersensitivity, consecutive intra-lateral ventricles injection of BIX02188 (0.1  $\mu$ g, 1  $\mu$ g, 10  $\mu$ g) before CCI and on days 1, 3, 5, and 7 after CCI were implemented. One hour after intra-lateral ventricles injection, behavior test was carried out. The result of PWT and PWL indicated that pre-treatment with BIX02188, not DMSO, could attenuate remarkably the mechanical allodynia and thermal hypersensitivity in a dose dependent manner ( $**P < 0.01$ ,  $*P < 0.05$ , BIX02188 (1  $\mu$ g, 10  $\mu$ g) +CCI vs DMSO+CCI group; Fig. 3B).

Intra-lateral ventricles injection of BIX02188 reduced the expression of p-ERK5

Western blot assay revealed that p-ERK5 in CSF-CN increased in CCI 14 day rats compared with sham group. Intra-lateral ventricles consecutive administration of BIX02188 every day, not DMSO, reduced the p-ERK5 expression in CSF-CN of CCI rats. However, the total ERK5 expression was not altered in any state (Fig. 4).



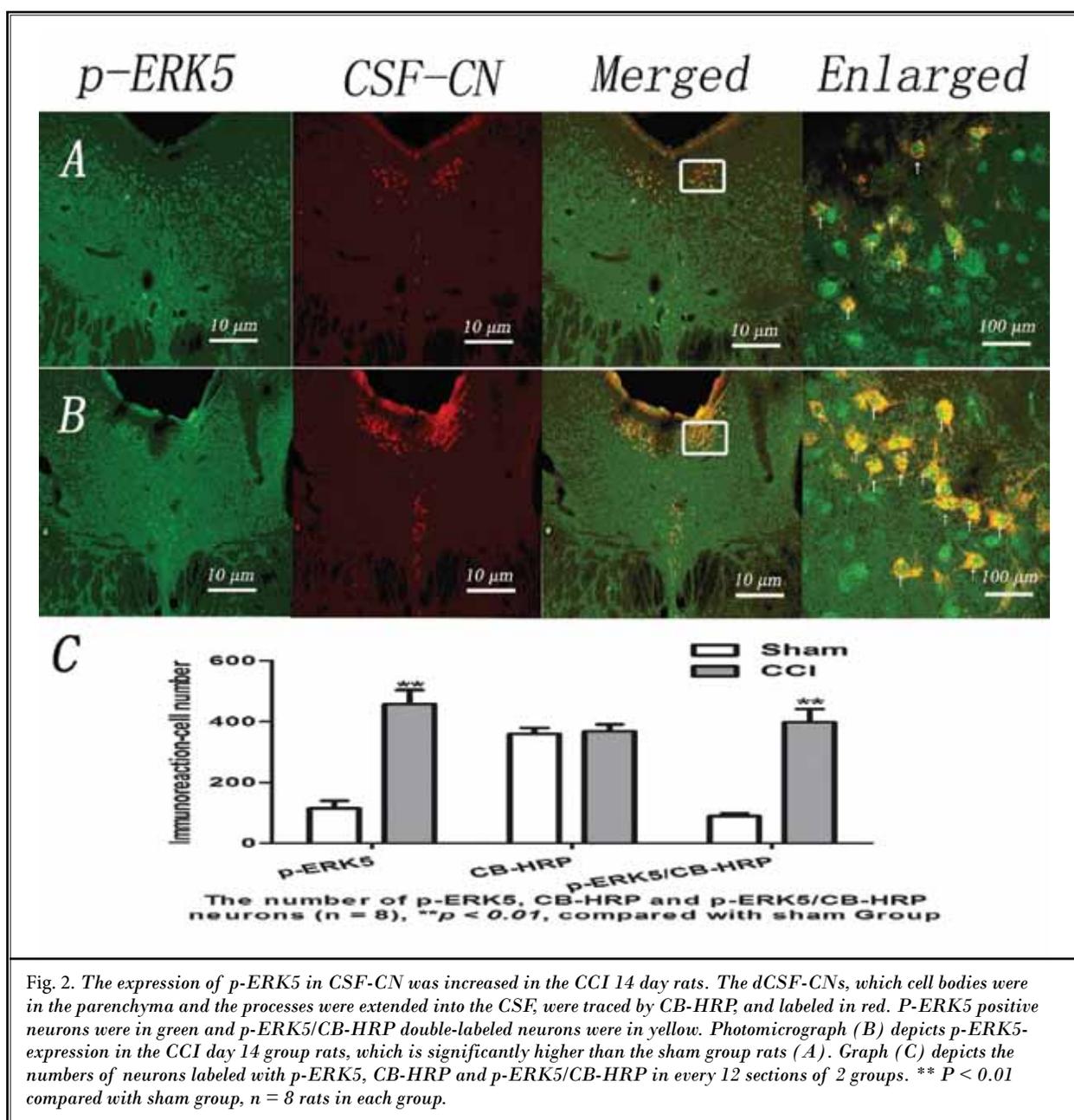


Fig. 2. The expression of p-ERK5 in CSF-CN was increased in the CCI 14 day rats. The dCSF-CNs, which cell bodies were in the parenchyma and the processes were extended into the CSF, were traced by CB-HRP, and labeled in red. P-ERK5 positive neurons were in green and p-ERK5/CB-HRP double-labeled neurons were in yellow. Photomicrograph (B) depicts p-ERK5-expression in the CCI day 14 group rats, which is significantly higher than the sham group rats (A). Graph (C) depicts the numbers of neurons labeled with p-ERK5, CB-HRP and p-ERK5/CB-HRP in every 12 sections of 2 groups. \*\* P < 0.01 compared with sham group, n = 8 rats in each group.

Expression of p-CREB was decreased by intra-lateral ventricles injection of BIX02188

Western blot result showed that p-CREB highly expressed in CSF-CN of CCI 14 day rats. Intra-lateral ventricles consecutive administration of BIX02188 every day, not DMSO, could significantly inhibit the increase of p-CREB. However, the total CREB expression was not altered in any state (Fig. 5).

## DISCUSSION

The dCSF-CNs are mainly located in the ventral PAG of the brainstem, and the bodies are located in the parenchyma of the brain and processes extend into the CSF in the cavity of the ventricle. According to their anatomical characteristics, the CSF-CN may play a vital function in the substance and signaling transport, modulating between the brain and CSF (10). In patho-

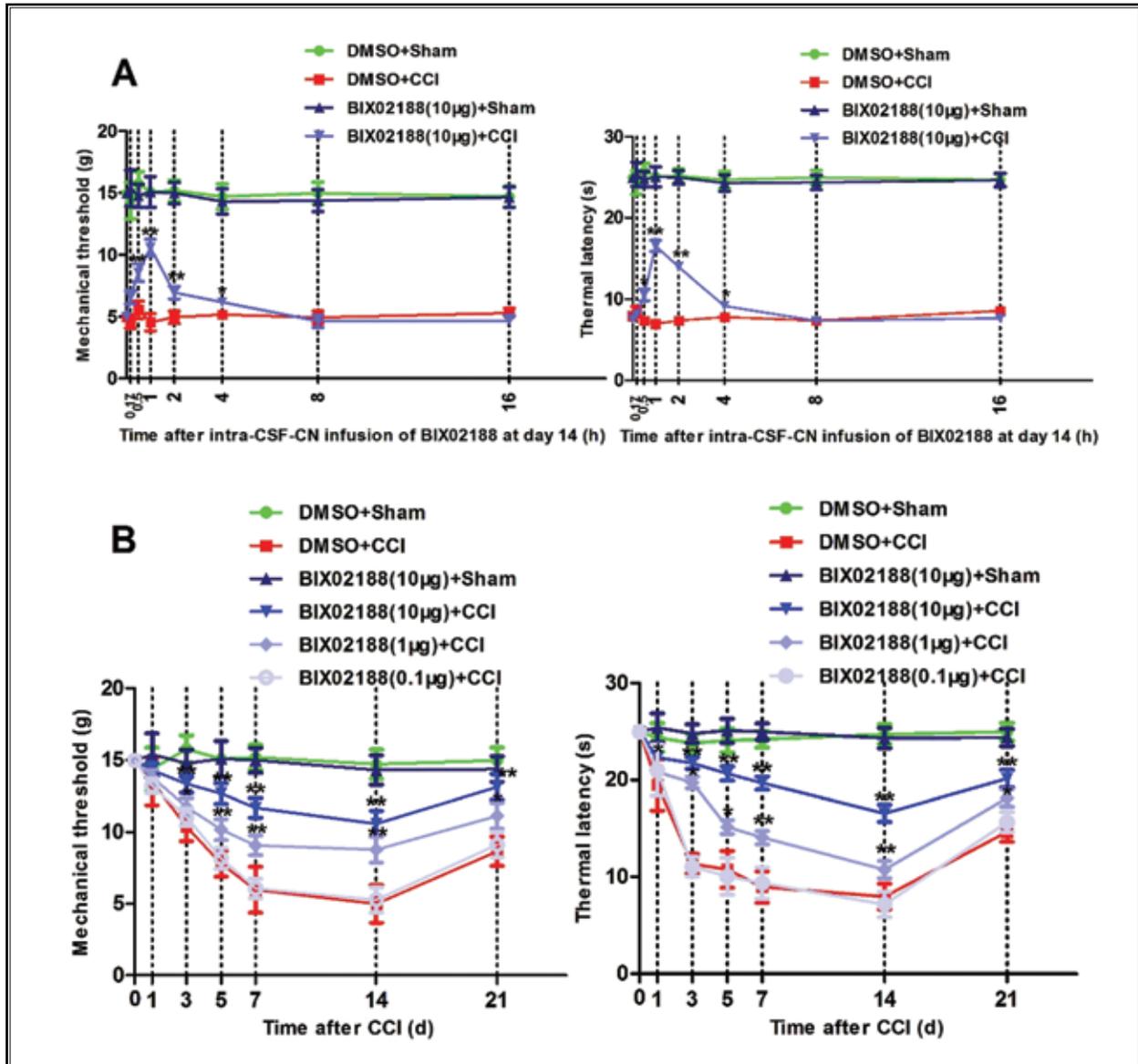


Fig. 3. Intra-lateral ventricles post- or pre-treatment with BIX02188 inhibited chronic constrictive injury-induced neuropathic pain. A: Post-treatment with BIX02188 (10 µg) reversed CCI-induced mechanical allodynia in paw-withdrawal threshold (PWT) test and thermal hyperalgesia in paw-withdrawal latency (PWL) test. One hour after intra-lateral ventricles infusion, the analgesic effect reached its peak, and then decreased gradually until hour 8. \*\*P < 0.01, \* P < 0.05 compared with DMSO+CCI group, n = 12 rats in each group. B: Pre-treatment of BIX02188 (0.1 µg, 1 µg, 10 µg) relieved CCI-induced mechanical allodynia and thermal hyperalgesia in a dose-dependent manner. \*\*P < 0.01, \* P < 0.05 compared with DMSO+CCI group, n = 12 rats in each group.

logical and certain special physiological conditions, the chemical composition of CSF can be changed, which was identified by clinical and animal experiments (10,24-25). Intercellular communication in the brain contains 2 types of transmission: wiring transmission by synaptic contacts and volume transmission, via the diffusion of

messengers by the intercellular space or CSF (25-26). Our previous study indicated that there were excitatory and inhibitory synapses between the CSF-CN and non-CSF-CN by electronic microscope technology (10). The actual composition of CSF may be influenced by dCSF-CNs for non-synaptic signal transmission via releasing or

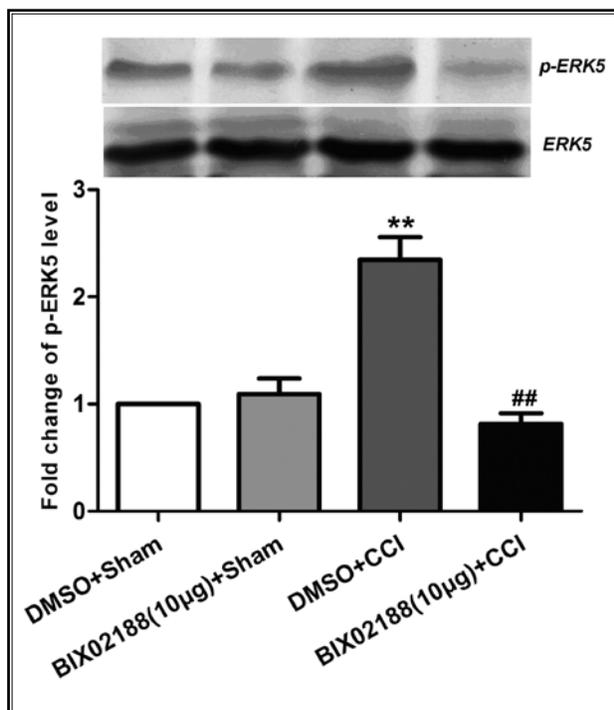


Fig. 4. Intra-lateral ventricles injection of BIX02188, not DMSO, inhibited p-ERK5 expression in CSF-CN of CCI rats. The representative western blot bands and the quantitative data for the expression of p-ERK5 and ERK5 in CSF-CN of CCI rats. The fold change for density of p-ERK5 bands normalized to total ERK5. Phosphorylation ERK5 level in DMSO+Sham group was set at 1 for quantifications. \*\*  $P < 0.01$ , compared with DMSO+Sham group, ##  $P < 0.01$  compared with DMSO+CCI group,  $n = 8$  rats in each group.

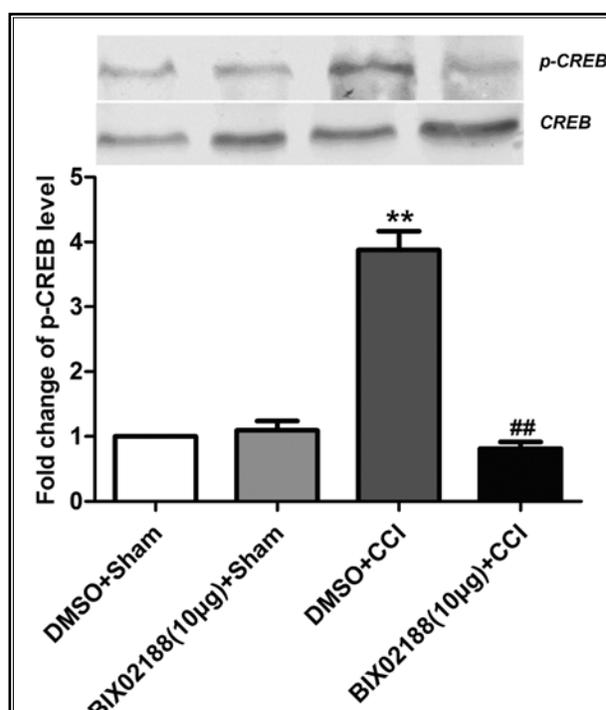


Fig. 5. Intra-lateral ventricles injection of BIX02188, not DMSO, inhibited p-CREB expression in CSF-CN of CCI rats. The representative western blot bands and the quantitative data for the expression of p-CREB and CREB in CSF-CN of CCI rats. The fold change for density of p-CREB bands normalized to total CREB. Phosphorylation CREB level in DMSO+Sham group was set at 1 for quantifications. \*\*  $P < 0.01$ , compared with DMSO+Sham group, ##  $P < 0.01$  compared with DMSO+CCI group,  $n = 8$  rats in each group.

absorbing bioactive substances (26). However, CSF-CN can transmit the information to neurons of other areas in the brain via synaptic contacts. In this study, we found CB-HRP/p-ERK5 double-labeled neurons were increased significantly in CSF-CN of rats with nerve injury, which indicated that p-ERK5 in CSF-CN might regulate the information transfer in pathological conditions between brain parenchymal and CSF.

Neuropathic pain, considered as a multifactor neurogenic disorder, is a major public health issue, which can be caused by physical damage of neurons, cancer, and other diseases (27). Central sensitizations such as synaptic and neuronal plasticity are the neurobiological basis of behavior hyperalgesia under neuropathic conditions (28-30). Activation of intracellular kinase cascades, postsynaptic membrane receptors, or ion channels, and intranuclear gene expression contributes to the induc-

tion, development, and maintenance of central sensitization, especially mitogen-activated protein kinases (16). Compelling evidence indicates that MAPK play an important role in the development and maintenance of neuropathic pain. Activation of ERK is required for neuronal plasticity, such as learning and memory, and it also plays an important role in the transmission of pain sensation (31). Synapses, as the connection of dCSF-CNs, are the key structure of information transmission. Many peptides relevant to neuropathic pain, such as SP and calcitonin gene-related peptide (CGRP), are released from synaptic vesicles of dCSF-CNs, which may interact with their receptors leading to an augmentation of the discharge and long-term potentiation in dCSF-CNs. These peptides and their receptors activate ERK/MAPK by coupling to the ERK/MAPK (32-33). Activated ERK/MAPK is translocated to nucleus and phosphorylate

nuclear factor p90 ribosomal S6 kinase (RSK) which then activates CREB (34-35). Neuropathic pain-related genes, such as brain-derived neurotrophic factor (BDNF), calcitonin gene-related peptide (CGRP), the immediate early gene c-Fos, neurokinin 1 receptor, and the alpha subunit of calcium-calmodulin-dependent kinase (CaMK), might be activated by cAMP CREB and involved in synaptic plasticity underlying neuropathic pain (36-39). Our previous study showed p-ERK5 might contribute to inflammatory pain by regulating the activation of CREB (8). In this research we found ERK5 was activated in CSF-CN in nerve injury rats; besides these, intra-lateral ventricles injection of BIX02188 attenuated CCI-induced mechanical allodynia and thermal hypersensitivity, which indicated that ERK5 in CSF-CN might be involved in neuropathic pain. However, considering selective inhibition of ERK5 activation only partially suppressed sensitization of pain, we presumed that neuropathic pain maybe a process involving a multi-signaling pathway, and other neurotrophic factors or small protein might contribute to it. In addition, in this

study, CCI-induced mechanical allodynia and thermal hypersensitivity significantly increased the activation of CREB in CSF-CN. However, intra-lateral ventricles injection of BIX02188 remarkably inhibited the expression of p-CREB in CSF-CN in CCI rats. Based on these findings, we proposed that activation of ERK5 might increase the expression of p-CREB in CSF-CN of neuropathic pain rats and the function of ERK5 might be partly regulated by CREB. These new findings extended our understanding about the role of ERK5 pathway in neuropathic pain.

## CONCLUSIONS

In summary, we found that activation of ERK5 in CSF-CN might contribute to the development of neuropathic pain. Our findings pointed to the potential blockade of ERK5 signaling in CSF-CN as a new therapeutic strategy for neuropathic pain and its specific pharmacological inhibitor BIX02188 could have potential use for alleviation of mechanical allodynia and thermal hypersensitivity in the future.

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