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Research Paper

Early intervention of ERK activation in the spinal cord can block initiation of peripheral nerve injury-induced neuropathic pain in rats

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Abstract: The present study is to investigate whether the extracellular signal-regulated kinase (ERK) and cAMP response element binding protein (CREB) signaling pathway contributes to the initiation of chronic constriction injury (CCI)-induced neuropathic pain in rats. Mechanical allodynia was assessed by measuring the hindpaw withdrawal threshold in response to a calibrated series of von Frey hairs. Thermal hyperalgesia was assessed by measuring the latency of paw withdrawal in response to a radiant heat source. The expressions of phosphor-ERK (pERK) and phosphor-CREB (pCREB) were examined using Western blot analysis and immunohistochemistry. An early robust increase in the expression of pERK on the spinal cords ipsilateral to injury was observed on day 1 after CCI, when the CCI-induced behavioral hypersensitivity had not developed yet. Moreover, the upregulation of pERK expression in ipsilateral spinal cord was associated with the increase in pCREB expression in bilateral spinal cord. Intrathecal administration of mitogen-activated protein kinase kinase (MEK) inhibitor U0126 before CCI can efficiently block and delay the CCI-induced mechanical allodynia and thermal hyperalgesia. These data suggest that activation of ERK and CREB in the spinal cord contributes to the initiation of peripheral nerve injury-induced pain hypersensitivity, and an early intervention strategy should be proposed.

Key words: allodynia; hyperalgesia; ERK-CREB signaling pathway; early intervention; spinal cord; rats

早期干预ERK在脊髓的激活可阻断外周神经损伤引起的神经病理性疼痛的发生

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摘 要：本文采用大鼠坐骨神经慢性压迫损伤引起的神经病理痛模型，研究脊髓背角细胞外信号调节激酶(extracellular signal-regulated kinase, ERK)在外周神经损伤引起的神经病理疼痛发生中的作用。结果显示，单侧坐骨神经压迫性损伤后1天，大鼠损伤侧脊髓背角ERK的磷酸化(激活)水平显著上调，其下游转录因子cAMP 反应原件结合蛋白(cAMP response element binding protein, CREB)在双侧脊髓背角的激活水平也同时上调，而此时由神经损伤引起的痛觉敏化行为尚未出现。神经损伤之前和损伤后早期鞘内给予促分裂原活化蛋白激酶激酶(mitogen-activated protein kinase kinase, MEK)的抑制剂U0126，可阻断和延迟坐骨神经损伤引起的触诱发痛和热痛觉过敏行为的发生。这些结果提示，脊髓背角ERK-CREB信号的激活参与外周神经损伤引起的神经病理疼痛的发生，对该信号通路的早期干预可能是控制神经病理性疼痛的重要手段。

关键词：触诱发痛；痛觉过敏；ERK-CREB信号通路；早期干预；脊髓；大鼠
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Neuropathic pain, refers to the pain caused by damage to nervous tissue, is a major clinical problem. Injury or permanent loss of primary afferent fibers (deafferentation) differentiates peripheral neuropathic pain from other types of pain. Peripheral nerve injury induced in various ways may produce chronic pain states characterized by positive sensory phenomena (spontaneous pain, allodynia, and hyperalgesia)[1]. These sensory phenomena are likely to have many underlying mechanisms, including ectopic generation of impulses as well as the *de novo* expression of neurotransmitters and their receptors and ion channels, involving a series of gene expressions. It has also been shown that injury to primary afferents can give rise to a global increase in excitability of dorsal horn neurons[2–5]. Accumulating evidence indicates that such process of "central sensitization" serves as a key mechanism in the development and maintenance of chronic pain, particularly neuropathic pain[6–8].

Extracellular signal-regulated kinase (ERK) belongs to a group of evolutionarily conserved serine/ threonine protein kinases that play critical roles in cell proliferation, differentiation, and survival[9]. The ERK is activated by dual phosphorylations on their regulatory tyrosine and threonine residues by an upstream kinase, mitogen-activated protein kinase kinase (MEK), which is activated by membrane depolarization and calcium influx[10]. Increasing evidence indicates that ERK had been linked to signal transduction cascades that regulate neuronal activity and plasticity[11, 12]. ERK activation is associated with the transcription factor cAMP response element binding protein (CREB) in cultured hippocampal neurons and brain slices. Phosphorylation of CREB (pCREB) at serine 133 activates cAMP response element (CRE)-mediated gene expression[13, 14]. ERK is activated (i.e., phosphorylated) in the dorsal horn of the spinal cord and in brain stem nuclei after peripheral somatic or visceral stimulation or inflammation. Activation of ERK in dorsal horn neurons contributes to pain hypersensitivity[15, 16], because of both a role in promoting acute central sensitization, an activity-dependent increase in the excitability of dorsal horn neurons[2, 17, 18], and alteration in gene transcription in the spinal cord[19]. ERK has been implicated in the enhanced excitability of spinal cord dorsal horn neurons in models of inflammatory pain[15, 19–23] and neuropathic pain models[24–28]. Intrathecal administration of U0126 [1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylmercapto) butadiene] or PD98059 [2-(2-amino-3-methoxyphenyl-4H-1-benzopyran-4-one], both inhibitors of MEK, attenuates pain hypersensitivity[15, 19, 22, 29, 30]. However, whether the activation of ERK and CREB occurs prior to behavioral hypersensitivity following peripheral never injury and the effect of early intervention on the initiation of neuropathic pain are not clear.

1 MATERIALS AND METHODS

1.1 *Animals*

Adult male Sprague-Dawley rats (200–220 g) from Experimental Animal Center, Fudan University were housed in groups (3–5 per cage) with free access to food and water and maintained on a 12:12 h light-dark cycle with a constant room temperature of (20 ± 2) ºC. Prior to surgery or behavioral tests, the animals were handled daily at least 3 days. All experiments were performed in according with the guidelines of the International Association for the Study of Pain and were approved by the Shanghai Animal Care and Use Committee. All efforts were made to minimize the number of animals used.

1.2 *Chronic constriction injury (CCI) of the sciatic nerve*

Rats were deeply anesthetized with Pentobarbital Sodium (80 mg/kg). The skin of right hind limb was sterilized with iodine tincture, then 75% alcohol, and the right sciatic nerve exposed at the mid-thigh level by blunt dissection of the biceps femoris. For CCI group rats, four chromic gut (4-0) ligatures were tied loosely around the nerve at about 1 mm apart, as described by Bennett and Xie[31]. For sham group rats, the sciatic nerve was isolated without ligation. After CCI or sham surgery, the overlying muscles and skin were closed respectively in layers with 4-0 silk sutures.

1.3 *Intrathecal cannula implantation and drugs delivery*

For intrathecal delivery, an intrathecal catheter (PE-10 tubing) was inserted through the space between the L4 and L5 vertebrae and extended to the subarachnoid space of the lumbar enlargement (L4 and L5 segments) under Pentobarbital Sodium (80 mg/kg) anesthesia. The catheter was filled with sterile saline (approximately 4 μL), and the external end was closed. The cannulated rats were allowed to recover for 3–4 days and were housed individually. Rats that showed any neurological deficits resulting from the surgical procedure were ex-

cluded from the experiments.

The MEK (ERK kinase) inhibitor U0126 (Sigma) was diluted in 20% DMSO (Sigma). The MEK inhibitor U0126 (3 μg/10 μL) and vehicle control (20% DMSO, 10 μL) was administered every day from 1 d pre-CCI to 3 d post-CCI immediately after behavioral tests.

1.4 *Behavioral tests*

Animals were habituated to the testing environment daily for at least 3 days before baseline test. The room temperature and humidity remained stable for all experiments. For von Frey filament test, the hind paw withdrawal threshold (PWT) was determined using a calibrated series of von Frey hairs (Stoelting Co., Wood Dale, IL) ranging from 1 to 26 g. Animals were placed individually into Plexiglas chambers with a customized platform that contains 1.5-mm diameter holes in a 5-mm grid of perpendicular rows throughout the entire area of the platform. After acclimation to the test chambers, a series of nine calibrated von Frey hairs were applied to the central region of the plantar surface of one hind paw in ascending order (1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g). A particular hair was applied until buckling of the hair occurred. This was maintained for 0.2 s. The hair was applied only when the rat was stationary and standing on all four paws. A withdrawal response was considered valid only if the hind paw was completely removed from the customized platform. Each hair was applied five times at 5 s intervals. If withdrawal responses did not occur more than twice during five applications of a particular hair, the next ascending hair in the series was applied in a similar manner. Once the hind paw was withdrawn from a particular hair in three of the five consecutive applications, the rat was considered responsive to that hair. The PWT was defined as the lowest hair force in grams that produced at least three withdrawal responses in five tests. After the threshold was determined for one hind paw, the same testing procedure was repeated on the other hind paw at 10-min interval.

Thermal hyperalgesia was assessed by measuring the latency of paw withdrawal (PWL) in response to a radiant heat source. Rats were placed individually into Plexiglas chambers on an elevated glass platform, under which a radiant heat source (model 336 combination units, IITC/Life Science Instruments, Woodland Hill, CA) was applied to the glabrous surface of the paw through the glass plate. The heat source was turned off when the rat lifted the foot, allowing the measurement of time from onset of radiant heat application to withdrawal of the hind paw. This time was defined as the hind paw withdrawal latency. The heat was maintained at a constant intensity, which produced a stable PWL of 10–12 s before CCI surgery. A 20-s cut-off was used to prevent tissue damage in the absence of a response. Both hind paws were tested for three trials at each time period with 10-min intervals between each trial. The average of the three trials was then determined.

The tests were performed on each of 3 successive days prior to surgery and alternate days up to 7 d after surgery.

1.5 *Immunohistochemistry*

After defined survival times, rats were given an overdose of urethane (2 g/kg, i.p.) and perfused intracardially with saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB, pH 7.4). The L4/5 segments of spinal cord was then removed, post-fixed in the same fixative for 4 h at 4 °C, and immersed from 10% to 30% gradient sucrose in PB for 24–48 h at 4 °C for cryoprotection. Transverse spinal cord was cut into 30 μm-thick segments in a cryostat and processed for immunofluorescence. After washing in phosphate buffer saline, the tissue sections were incubated in phosphate-buffered saline (PBS) containing 10% normal donkey serum and 0.3% Triton X-100 at room temperature for 2 h, followed by primary polyclonal mouse anti-pERK (1:3 000, Sigma) and rabbit anti-pCREB (1:3 000; Upstate Biotechnology) antibody at 4 °C for 48 h in PBS with 1% normal donkey serum and 0.3% Triton X-100. After three 10-min rinses in PBS, the sections were then incubated in FITC- or rhodamine-conjugated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch) for 2 h at 4 °C, then washed in PBS. For double immunofluorescence, spinal sections were incubated with a mixture of pERK (1:3 000) and pCREB (1:3 000) over night at 4 °C, followed by a mixture of FITC- and rhodamine-conjugated secondary antibodies for 2 h at room temperature. All sections were coverslipped with a mixture of 50% glycerin in PBS, and then observed with Olympus laser-scanning microscope.

1.6 *Western blot analysis*

After defined survival times, rats were killed by overdose of urethane (2 g/kg, i.p.). The L4–5 spinal cord was rapidly removed. Then the spinal segment was cut

into a left and right half from the ventral midline. Finally, the right half was further split into the dorsal and ventral horn at the level of the central canal. After dissection, all tissues were rapidly frozen in liquid nitrogen and stored at −70 °C until further processing. Frozen spinal cords were directly homogenized in lysis buffer (12.5 μL/mg tissue) containing protease inhibitors (Roche) and PMSF (Sigma). After 10 000 r/min centrifugation for 15 min at 4 °C, supernatant was used for Western blotting.

Equal amount of protein was loaded and separated in 10% Tris-Tricine SDS-PAGE gel. The resolved proteins were transferred onto PVDF membranes (Millipore). The membranes were blocked in 5% non-fat milk for 2 h at room tepmperature, and incubated overnight at 4 °C with mouse anti-pERK (1:3 000, Sigma) primary antibody. The blots were then incubated with the secondary antibody, goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) (1:1 000, Pierce) for 2 h at 4 °C. Signals were finally visualized using enhanced chemiluminescence (ECL, Pierce) and the blots were exposed onto X-films. All Western blot analysis was performed at least three times, and consistent results were obtained.

1.7 *Statistics*

All data were expressed as mean ± SEM. For behavioral tests, pre- and post-treatment of PWTs and PWLs were analyzed by two way (treatment × time) ANOVA. For the quantification of Western blot signals, the density of pERK bands was measured with a computer-assisted imaging analysis system (Adobe). The same size square was drawn around each band to measure the density and the background near that band was subtracted. Relative pERK levels were presented by p/tERK ratio. GAPDH serves as loading control. Differences between groups were compared using one way ANOVA followed by Dunnett. For the quantification of immunoreactive signals, 5–6 spinal cord sections per animal were selected. For each section, total area of positive neurons in the spinal cord dorsal horn was measured by Image J. The criterion for statistical significance was $P < 0.05$.

2 RESULTS

2.1 Activation of ERK in the lumbar spinal cord following CCI

As a major subfamily of mitogen-activated protein kinase (MAPK), ERK has been proved by accumulating evidence that it is involved in pain sensitization after tissue inflammation and injury via distinct molecular and cellular mechanisms. In order to investigate the role of ERK in early phase of neuropathic pain, CCI of sciatic nerve was used for the neuropathic pain model. By 2 d after CCI surgery, persistent mechanical allodynia and thermal hyperalgesia were observed (Fig. 1 *A* and *B*). Compared with sham group, PWT and PWL in the ipsilateral side of the CCI rats significantly decreased. No mirror pain appeared during the whole behavioral test period.

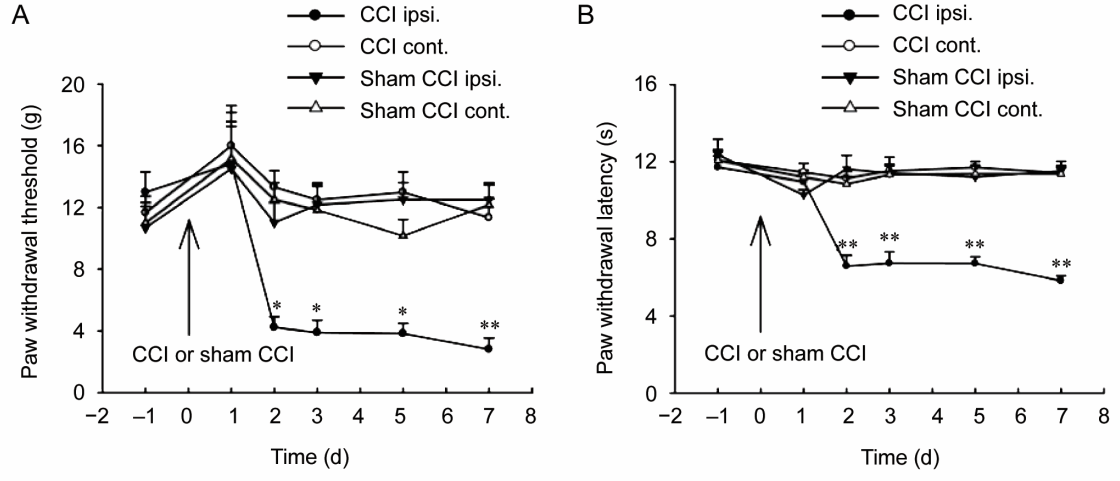

Fig. 1. Chronic constriction injury (CCI) induced mechanical allodynia and thermal hyperalgesia. *A*: Mechanical allodynia was assessed by measuring the paw withdrawal threshold (PWT); *B*: Thermal hyperalgesia was assessed by measuring the paw withdrawal latency (PWL). Mean ± SEM, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* sham CCI (ipsilateral to CCI).

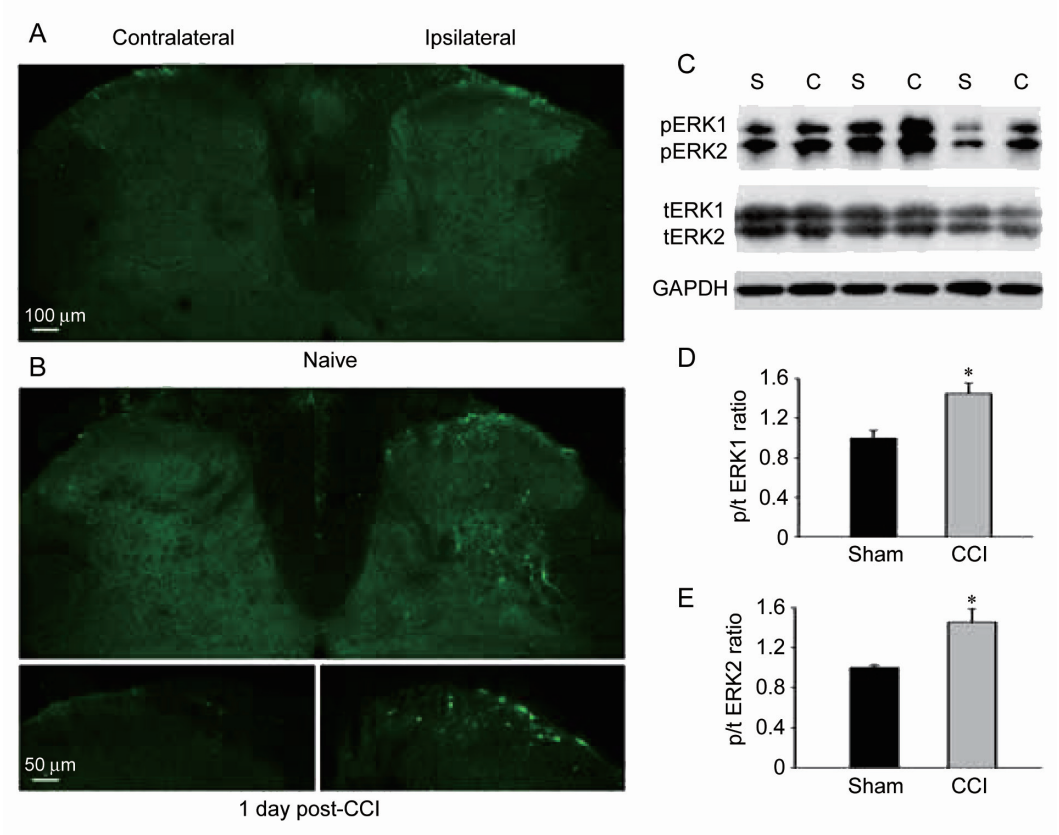

Fig. 2. CCI-induced activation of ERK on the lumbar spinal cord. Immunohistochemistry showing expression of pERK on the spinal dorsal horn in naive control (*A*) and CCI (*B*) rats. *C*: Western blot showing increased pERK level in the ipsilateral lumbar spinal cord on day 1 after CCI. Abbreviation: S, sham; C, CCI. GAPDH served as loading control. Quantification of pERK1 (*D*) and pERK2 (*E*) level on day 1 after sham and CCI surgery. Mean ± SEM, $n = 3$. $^{*}P < 0.05$ *vs* CCI.

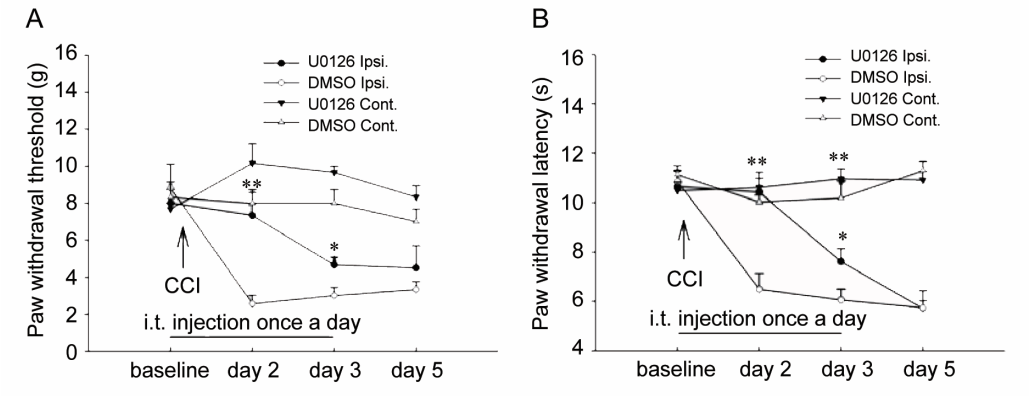

Fig. 3. Blockade of ERK activation delayed the development of CCI-induced mechanical allodynia (*A*) and thermal hyperalgesia (*B*). U0126 was applied intrathecally once daily for 4 days with the first injection at one day before CCI. Mean ± SEM, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* DMSO (ipsilateral to CCI).

To determine whether ERK signaling pathway in the spinal cord is involved in the early phase of neuropathic pain, we examined the activation of ERK in the lumbar spinal cord at 1 day after CCI, when the CCI-induced behavioral hypersensitivity had not developed yet. The results revealed that CCI resulted in the induction of pERK in the ipsilateral spinal dorsal horn to injury (Fig. 2 *A* and *B*). This result was further confirmed

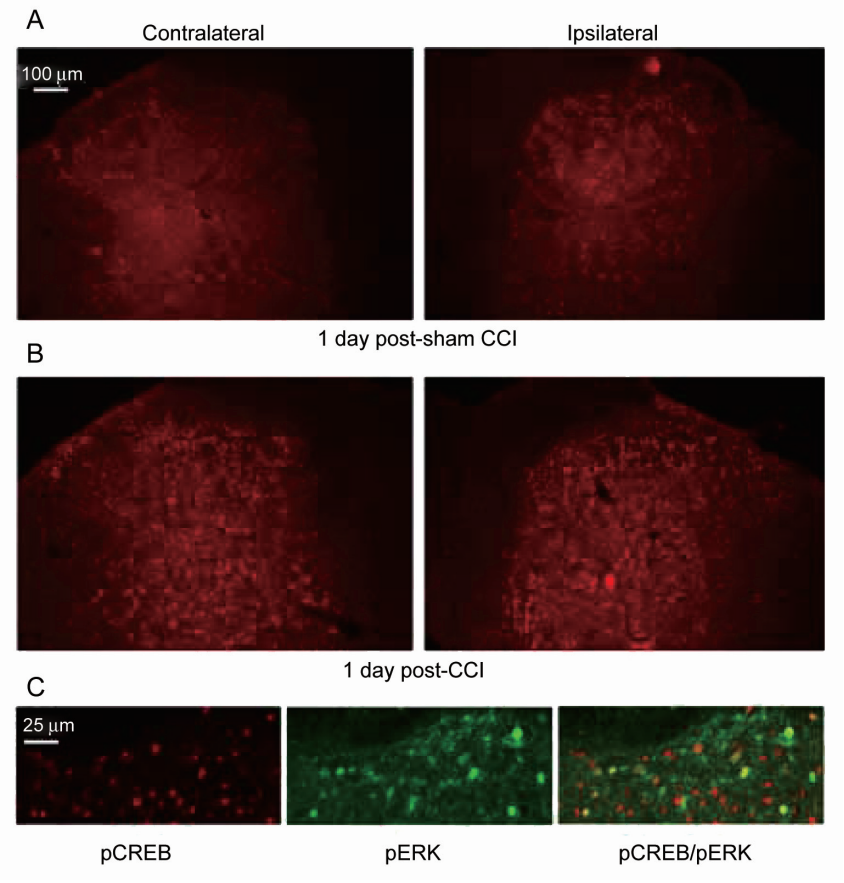

Fig. 4. CCI-induced increase in pCREB expression on the lumbar spinal cord. Immunohistochemistry showing expression of pCREB on the spinal dorsal horn on day 1 after sham CCI (*A*) and CCI (*B*). *C*: Double staining showing the co-localization of pERK- and pCREB-positive cells in the ipsilateral superficial spinal dorsal horn.

by Western blot analysis (Fig. 2*C*–*E*).

2.2 Blockade of ERK activation in the lumbar spinal cord prevents the initiation of pain hypersensitivity

To investigate the role of ERK activation in initiation of CCI-induced pain hypersensitivity, we administered MEK inhibitor, U0126 once daily for 4 days with the first injection at 1 day pre-CCI operation. When repeated DMSO was applied intrathecally from 1 day pre-CCI to 3 days post-CCI, robust mechanical allodynia and thermal hyperalgesia appeared in the ipsilateral hindpaw to injury on day 2 after CCI. In contrast, repeated administration of U0126 significantly blocked and delayed the development of CCI-induced allodynia and thermal hyperalgesia (Fig. 3*A* and *B*).

2.3 Activation of CREB in the lumbar spinal cord following CCI

ERK activates a variety of transcription factors that in turn regulate the expression of many target genes[32]. The nuclear CREB is a key downstream transcription factor of ERK signaling. It is known that in NGF-stimulated PC12 cells, CREB is phosphorylated by ERK[33, 34]. We observed a correlation between ERK activation and CREB phosphorylation following CCI. The immunohistochemical results showed that the expression level of pCREB in the spinal dorsal horn robustly increased on day 1 after CCI (Fig. 4*A* and *B*). Double immunofluorescence staining indicated that the most pERK-positive cells were also pCREB-positive cells (Fig. 4*C*).

3 DISCUSSION

The ERK, one key member of the MAPK family, transduces a broad range of extracellular stimuli into diverse intracellular responses by producing changes in the level of gene expression or transcription. Accumulating studies show that ERK activation in spinal cord was induced by various noxious stimuli[15, 35]. Consistent with

behavioral pain hypersensitivities, subcutaneous injection of scorpion venom[36], formalin and capsaicin[15] induced a rapid and transient ERK activation, while complete Freund's adjuvant (CFA) inflammation[19], spinal cord injury[37], partial sciatic nerve ligation[25], spinal nerve ligation[26, 38, 39] and CCI of sciatic nerve[28] induced a persistent ERK activation in the spinal cord. It was also shown that blockade of spinal ERK activation by U0126 significantly inhibited bee venom-induced persistent spontaneous nociception, mechanical and heat hypersensitivities[40]. The distribution of activated ERK in different cell types on the spinal dorsal horn showed that acute inflammatory pain-induced pERK expressed only in neurons, but nerve injury induced a sequential activation of ERK in dorsal horn neurons, microglia and then astrocytes[15, 19, 27]. Thus, there might be different mechanisms underlying ERK activation in different pathological pain models.

The transcription factor CREB is one of the most important potential effectors of ERK related to central sensitization since many CREB-related genes, such as immediate early gene c-fos, BDNF, CGRP, the alpha subunit of CaMKII, and the neurokinin 1 receptor may contribute to central sensitization[41].

A previous study showed that both pERK1 and pERK2 were increased from 3–15 days after CCI[28]. In the present study, we demonstrated that CCI-induced ERK activation occurred even at the first day, an earlier period, before the initiation of behavioral hypersensitivity, confirming the Zhuang *et al.*'s report in another neuropathic pain model[26]. Furthermore, blockade of ERK activation during early phase of CCI significantly delayed the development of mechanical allodynia and thermal hyperalgesia in the present study, suggesting a strategy of early intervention by ERK inhibitors.

Another major member of MAPKs, p38, in the spinal dorsal horn also plays important roles in the pain hypersensitivity following tissue and nerve injury[42-44]. Activated p38 (p-p38) is increased in the spinal cord in various neuropathic pain models[45-48], and intrathecal administration of p38 inhibitors attenuates neuropathic pain[44, 49, 50]. Also, Cui *et al*. reported that bee venom-induced ERK activation peaked at 2 min, whereas the peak of p38 activation appeared at 1–3 days, lasted at lease 7 days when pain hypersensitivity has disappeared. Moreover, activated ERK mainly expressed in neurons, while p38 was activated in microglia, suggesting ERK and p38 MAPKs may play different roles in the development of bee venom-induced inflammatory pain[44].

Different from ERK activation in the ipsilateral superficial spinal cord to injury, pCREB expression was increased bilaterally in all lamina of the spinal cord following peripheral never injury, which consists with reports from Song *et al*.[28] and Ji *et al.*[51]. It was speculated that signal strength might affect gene expression differentially with respect to protein phosphorylation[51] or different intracellular signal transduction pathways contribute to pCREB expression in the ipsilateral and the contralateral spinal cords[28]. In the present study, we observed double staining cells of pERK and pCREB in the ipsilateral superficial spinal cord to CCI, suggesting that the CCI-induced CREB activation in the spinal cord was, at least in part, mediated by pERK, and an earlier activation of both them was involved in the initiation of neuropathic pain.

Zhuang *et al.*[26] found that there was a sequential activation of ERK in the spinal cord after L5 spinal nerve ligation (SNL), first in neurons, then in microglia, and finally with a delay of several weeks, in astrocytes. However, the further detailed investigation and exploration on the mechanisms underlying involvement of ERK-CREB signal pathway in neuropathic pain need to be performed.

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