Animal Study

EphrinB-EphB Signaling Induces Hyperalgesia through ERK5/CREB Pathway in Rats

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Background: There are numerous studies implicating that EphB receptors and ephrinB ligands play important roles in modulating the transduction of spinal nociceptive information. EphrinB-EphB signaling may contribute to hyperalgesia via various kinds of downstream molecules, the mechanisms of which have not been completely understood.

Objective: The aim of the present study was to identify whether ephrinB-EphB signaling could contribute to hyperalgesia through ERK5/CREB pathway.

Study Design: Controlled animal study.

Setting: University laboratory.

Methods: This study attempted to detect the changes of pain behaviors and the protein level of p-ERK5 and p-CREB by activating EphB receptors in the spinal cord of rats. To further confirm our hypothesis, we designed LV-siRNA for knockdown of spinal ERK5. When ERK5 was inhibited, we recorded the changes of spinal p-CREB expression and the pain behaviors of rats after activating EphB receptors. We also confirmed this conclusion in rat CCI model. Statistical analyses were performed using GraphPad Prism 5.

Results: Intrathecal injection of ephrinB2-Fc in rats evoked thermal hyperalgesia and mechanical allodynia, along with activation of ERK5 and CREB in the spinal cord. Knockdown of ERK5 inhibited ephrinB2-Fc-induced CREB activation and hyperalgesia. Blocking EphB receptors prevented CCI-induced neuropathic pain and spinal ERK5/CREB activation.

Limitations: More underlying mechanisms that underlie the relationship between ephrinB-EphB signaling and ERK5/CREB pathway will need to be explored in future studies.

Conclusions: Our study suggests that ERK5/CREB pathway plays important roles in the transduction of nociceptive information associated with ephrinB-EphB signaling. This study provides further understanding of the downstream mechanisms of ephrinB-EphB signaling and helps to explore new targets for treating pathological pain.

Key words: EphrinB-EphB signaling, MAPK, ERK5, CREB, hyperalgesia, pain, CCI, NMDA

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The Eph receptors are the largest subfamily of receptor tyrosine kinases (RTKs), which transmit external signals to the inside of cells. The Ephs consist of 13 members—A subclass (EphA1-8) and B subclass (EphB1-4, EphB6) (1). EphB receptors and their ligands, known as ephrinBs, play key roles in modulating many kinds of physiological and pathological processes, such as inflammation response and neuronal survival (2). Song et al showed that ephrinB-EphB receptor signaling contributes to hyperalgesia induced by neuropathic pain via regulating neural excitability and synaptic plasticity (3). They later proved

that EphB1 receptor is essential for the formation of long-term potentiation at synapses between primary sensory neurons and spinal dorsal horn neurons (4). There are other researches elaborating the vital role of EphB receptors in modulating inflammatory and neuropathic pain (5,6). Our previous studies have also demonstrated that ephrinB-EphB signaling in the spinal cord could regulate nociceptive process and contribute to central sensitization (7). However, the downstream mechanisms of ephrinB-EphB signaling are still not completely understood.

Mitogen-activated protein kinases (MAPK) transduce extracellular stimuli into intracellular responses and could regulate diverse physiological and pathological processes (8, 9). The MAPK family consists of extracellular signal-regulated protein kinase 1/2 (ERK1/2), p38, c-jun N-terminal kinase (JNK), and extracelluar signal-regulated protein kinase 5 (ERK5) (10). Many reports have shown that ERK1/2, p38, and JNK are involved in ephrinB-EphB signaling-induced pain hypersensitivity and neuronal plasticity (11-14). Recently, increasing studies have reported that ERK5, also known as big mitogen-activated protein kinase 1, also takes part in mediating the transduction of pain signals and contributes to hyperalgesia and allodynia after peripheral inflammation or nerve injury (15,16). ERK5 is specifically phosphorylated and activated by MEK5 (MAPK kinase 5). After phosphorylation, p-ERK5 translocates to the nucleus, activates several nuclear factors, and adjusts the downstream gene expression (15).

cAMP response element binding protein (CREB), a transcription factor, is one of the downstream targets of ERK5 (17). Activated CREB binds to the cAMP-response element sites (CRE) in the promoter regions of the DNA and initiates the transcription of some pain-related genes, which could contribute to the central sensitization associated with persistent pain states (18,19). In this study, we investigated whether ephrinB-EphB signaling could induce hyperalgesia via ERK5/CREB pathway in rats.

Methods

Animals

Adult male Sprague-Dawley rats (200-250g) were purchased from the Experimental Animal Center of Zhejiang University. The rats were kept under 12hr./12hr. light–dark cycle and a fixed room temperature (RT) of 23 ± 1°C. They had free access to food and water, and were housed more than 7 days before experimentation. All experiments were performed according to the regulations of International Association for the Study of Pain and were approved by Zhejiang Animal Care and Use Committee.

Intrathecal Drug Administration

Intrathecal injection was performed under inhalational anesthesia. The rat was placed in a plexiglas observation chamber, into which 1.375% isoflurane and one l/min. flow of oxygen was continuously delivered until the rat lost its righting reflex. Afterwards, the rat was taken out and placed in a nose cone for continued isoflurane administration. The lower back of the rat was shaved and sterilized. The intrathecal injection procedure was operated via lumbar puncture at the intervertebral space of L4–5. When the rat showed a sudden slight flick of the tail, which indicated that the cannula had entered into the subarachnoid space, the drugs were slowly injected into the subarachnoid space within 30 seconds. After injection, the cannula was held fixedly for a further 10 seconds to prevent drug outflow. The whole procedure was to be completed within 30 minutes in case of respiratory depression of the rats. All reagents used in our study for intrathecal administration include ephrinB2-Fc chimera, EphB1-Fc chimera, and human IgG Fc fragment. EphrinB2-Fc chimera, which binds to and activates EphB1–B4 receptors, was purchased from Sigma (E0778). EphB1-Fc chimera (sc-9319, Santa Cruz Biotechnology) could bind to and inhibit ephrinB1-3. The human IgG Fc fragment was used as the control (ab206214, Abcam). These drugs were dissolved in Phosphate Buffer Solution. The drug doses each rat received are listed as follows: ephrinB2-Fc, 5μg in 10μl PBS; EphB1-Fc, 10 μg in 10 μl PBS; control Fc for ephrinB2-Fc and EphB1-Fc, 5 μg and 10 μg in 10 μl PBS. We excluded rats with motor dysfunction from the experiment.

Measurement of Mechanical Hyperalgesia

Mechanical allodynia was measured by paw withdrawal threshold (PWT). Rats were placed individually in transparent plastic cages with wire mesh bottom and were allowed to adapt to the environment for 30 minutes. We used the von Frey filament (began with 2g) to touch the plantar surface of the rat's left hind paw for 6 seconds, and then marked the paw withdrawal or paw licking response. If the rats showed a positive response, we switched to a lower filament. If the response was negative, a higher filament was used. The PWT value was obtained using the nonparametric method of

Dixon, which was described by Chaplan et al (20). On each rat, we performed 3 measurements; the average PWT value was taken as the final PWT.

Measurement of Thermal Hyperalgesia

Thermal hyperalgesia was measured by paw withdrawal latency (PWL). Rats were placed individually in transparent plastic cages and were allowed to adapt to the environment for 30 minutes. While the rat was in a motionless state, a radiant heat source was applied onto the plantar surface of the rat's left paw, through the glass plate. The heat was kept at a constant intensity. Once the rat showed paw withdrawal or paw licking response, the radiant heat source was immediately ceased. We recorded the total irradiating time as the PWL value. If the rat showed no positive response until 30 seconds, we cut off the radiant heat to prevent tissue damage and recorded 30 seconds as the PWL value. The process was performed according to the Hargreaves' test (21). On each rat, we performed 3 measurements with an interval of 5 minutes; the average PWL was taken as the final PWL.

Western Blotting

On deeply anesthetized rats, the spinal cord of lumbosacral enlargement (L4–5 segments) was quickly extracted and immediately stored in liquid nitrogen. Samples were homogenized in lysis buffer, which contains phenylmethylsulfonyl fluoride (100:1), for 30 minutes on ice. Then, they were centrifuged at 10,000 rpm for 15 minutes at 4°C. We collected the supernatants of the samples and estimated their protein concentration according to the Bradford method (22). After being heated at 100°C for 5 minutes and mixed with 1×loading buffer, a certain amount of each liquid sample (with equal protein amounts) and the marker (26616, Thermo Scientific) were loaded and electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel. We transferred the separated proteins onto polyvinylidene difluoride (PVDF) membranes at 300 mA for 60-90 minutes (depending on the molecular weight of our target protein). The membranes were then blocked in 10% non-fat dry milk for one hour at room temperature. According to the marker and the molecular weight of each target protein, we scissored the membranes and they were separately incubated overnight at 4°C with rabbit anti-ERK5 antibody (ab196609, 1:1000, Abcam), rabbit anti-p-ERK5 (ab5686, 1:1000, Abcam), rabbit anti-CREB (ab32515, 1:500, Abcam), rabbit anti-p-CREB (ab32096, 1:500, Abcam), and mouse anti-GAPDH (60004-1-lg, 1:1000, Proteintech) primary antibodies. After primary incubation, the membranes were taken out and extensively washed for 3×5 minutes with TBST (Tris-buffered saline Tween-20). Then, p-ERK5, ERK5, p-CREB, and CREB were incubated with secondary antibody — goat anti-rabbit peroxidase (HRP, 1:5000; A0208, Beyotime) at room temperature. GAPDH was incubated with goat anti-mouse peroxidase (HRP, 1:5000, RS0001, Ruiying Biological) at room temperature. After 2 hours, the membranes were taken out again and extensively washed for 3×5 minutes with TBST. The protein signals were detected using enhanced chemiluminescence. Quantity One 4.6.2 (Bio-Rad,USA) was used to perform Western blot densitometry analysis.

Immunohistochemistry

After being deeply anesthetized by sodium pentobarbital, the rats were transcardially perfused with 0.9% sodium chloride, and then with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). After perfusion, the L5 spinal cord segments were removed and fixed in paraformaldehyde overnight. Later, the paraformaldehyde was then replaced with 30% sucrose overnight. In a cryostat, transverse spinal cord sections of L5 (10μm) were cut onto the glass slides, and 10–16 sections were randomly selected from each sample. Those sections were incubated in blocking solution, which consists of 5% BSA and 0.3% Triton X-100, at room temperature for 2 hours. For double immunofluorescence, spinal sections were incubated with a mixture of rabbit anti-pERK5 (1 : 200) antibody and mouse anti-Iba1 (microglia marker, sc-32725; 1 : 200; Santa Cruz Biotechnology) antibody, or mouse anti-GFAP (astrocyte marker, sc-33673; 1 : 200; Santa Cruz Biotechnology) antibody, or mouse anti-NeuN (neuron marker,sc-33684;1:200; Santa Cruz Biotechnology) antibody at 4°C overnight. After primary incubation, the spinal sections were washed for 3×5 minutes in PBS and were then incubated in a mixture of fluorescein-congugated donkey anti-rabbit secondary antibody (A0453, 1:500; Beyotime) and goat anti-mouse secondary antibody (A0428, 1:500; Beyotime) for 2 hours away from light and at room temperature. Then, the sections were washed in PBS again, for 3×5 minutes, in a dark box to keep them away from light. Finally, a covership was adhered onto each glass slide. The images of the sections were examined using a fluorescence microscope (Olympus).

Lentivirus Construction and siRNA Transfections

For targeted knockdown of ERK5, 3 small interfering RNAs (siRNAs) targeting the complementary DNA (cDNA) sequence of rat ERK5 were designed and synthesized by Obio Technology (Shanghai) company (www.oobio.com.cn). The nucleotide sequences were: siRNA1 (Y2264): 5′-GCCGCTCACACTAGAACATGT-3′, siRNA2 (Y2265): 5′-GCGCATTAAGGAGGCCATTGT-3′, and siRNA3 (Y2266): 5′-GCTTTGACCTGGAGGAATTCT-3′. A scrambled sequence was also designed as a negative control (NC, Y006): 5′-TTCTCCGAACGTGTCACGT-3′. The cDNAs corresponding to these 3 siRNAs and NC were subcloned into a lentivirus vector. The resulting recombinant lentiviral vectors were designated as LV-siERK5 1, LV-siERK5 2, siERK5 3, and LV-NC. Each titer was listed as follows: LV-siERK5 1 (Y2264), $2.73*10^8$ TU/ML; LV-siERK5 2 (Y2265), $3.01*10^8$TU/ML; LV-siERK5 3 (Y2266), $3.82*10^8$TU/ML; LV-NC (Y006), $2.54*10^9$TU/ML. On each rat, the LV-siERK5 and LV-NC were administrated intrathecally for 3 consecutive days (10μl/d). Then, the effect of ERK5 knockdown was analyzed by western blotting with antibody to ERK5 in rat L4-5 spinal cord, which were detected on 1 days, 3 days, 5 days, 7 days, 9 days, 11 days, 13 days, and 15 days after 3 consecutive days of injection (n=6 for each LV-siRNA at different time points).

Model of Neuropathic Pain

A model of chronic constrictive injury (CCI), which produced peripheral nerve injury in rats, was performed in our study (23). We shaved the fur and sterilized the skin of the rats' left hind limbs with iodine tincture. We cut the skin at the mid-thigh level and bluntly dissected the biceps femoris, exposing the left sciatic nerve. Three silk threads (4-0) were tied around the nerve with a one mm interval. In sham surgery group, we isolated the nerve but did not tie it. After surgery, the dissected muscles and skin were sutured in turn and were sterilized.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). We used Student's t-test to compare the data between 2 groups of samples. To compare the data among more than 2 groups of samples, we used the one-way repeated analysis of variance (ANOVA) and two-way repeated ANOVA, followed by post hoc analysis (Bonferroni test). All data were expressed as mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

Results

1. EphrinB2-Fc induced thermal and mechanical hyperalgesia, as well as spinal ERK5 and CREB activation.

After intrathecal injection of ephrinB2-Fc (agonist of EphB receptors) in rats, a decrease occured in both thermal and mechanical pain threshold at 2 hours, and a significant decrease occured at 4 hours, compared with the control group (Fc injection). The thermal hyperalgesia could last more than 48 hours. The mechanical hyperalgesia lasted up to nearly 48 hours (Fig. 1A and 1B). Previous studies have shown that activation of EphB receptors could activate some of the MAPK members (11,24). In the present study, we wanted to figure out whether ERK5, a member of the MAPKs, could be activated by activating EphB receptors. Western blotting analysis showed that intrathecal injection of ephrinB2-Fc caused a time-dependent increase in p-ERK5 expression in the spinal cord. The activation of EphB receptors promoted the phosphorylation of ERK5 (Fig. 1C and 1D). During the time course, p-ERK5 expression increased at 2 hours after injection and exerted a peak level at 4 hours, which was consistent with the behavioral result above. The same effect also occurred on the expression of CREB and p-CREB after ephrinB2-Fc injection, as shown in Fig. 1C and 1D.

2. ERK5 was activated in microglia, but not in neurons or astrocytes after intrathecal injection of ephrinB2-Fc.

Some studies have shown that spinal activation of ERK5 induced by nerve injury was mainly in microglia (25). To investigate the type of cell in which the activation of ERK5 caused by ephrinB2-Fc was located, we took out L5 spinal cord segments from rats at 4 hours after ephrinB2-Fc injection and performed double immunostaining of p-ERK5 and Iba1 (microglia marker), NeuN (neuron marker), and GFAP (astrocyte marker). As shown in Fig. 2A-2C, a large amount of p-ERK5-immunoreactive cells and the microglia marker Iba1 colocalized in the spinal cord. P-ERK5 did not colocalize with NeuN or GFAP. These results indicated that intrathecal injection of ephrinB2-Fc induced ERK5 activation in microglial cells, rather than in neurons or astrocytes in the spinal cord.

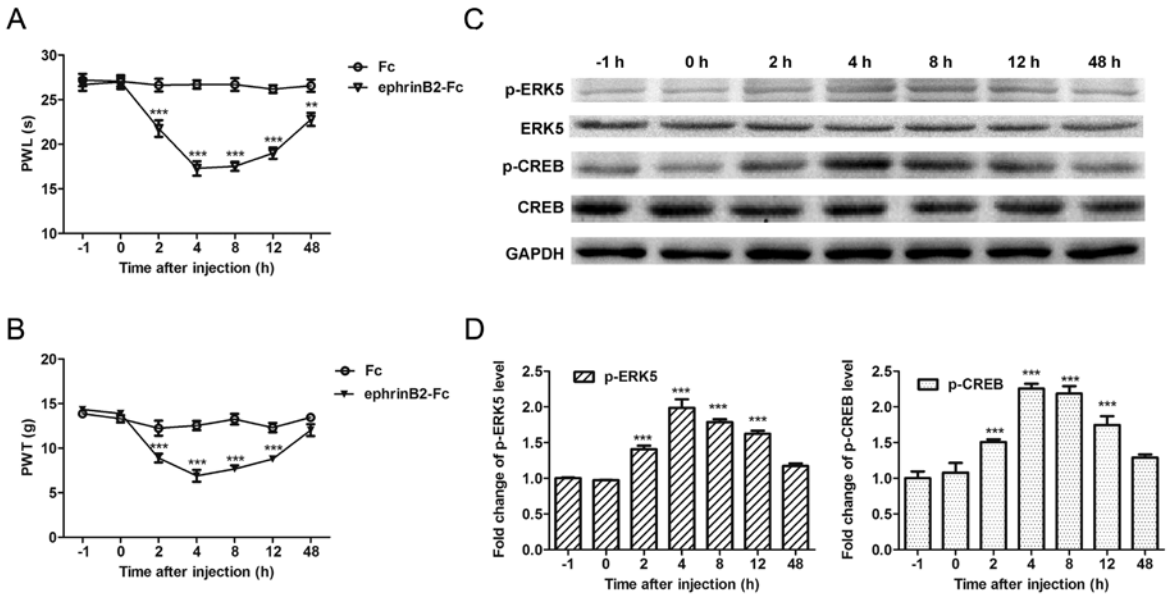

Fig. 1. *The effect of ephrinB2-Fc on thermal and mechanical hyperalgesia, and spinal ERK5 and CREB activation. (A-B): PWL and PWT were measured at -1 hr., 0 hrs., 2 hrs., 4 hrs., 8 hrs., 12 hrs., and 48 hrs. after ephrinB2-Fc (5μg i.t.) injection. Data were expressed as mean ± SEM. **P < 0.01, ***P < 0.001, compared with control group (Fc 5μg i.t.); n=8 in each group. (C): p-ERK5, ERK5, p-CREB, and CREB protein levels in the spinal cord at 1h before injection, right after injection, and 2 hrs., 4 hrs., 8 hrs., 12 hrs., and 48 hrs. after injection of ephrinB2-Fc (5μg i.t.). (D): The fold change for the density of p-ERK5 normalized to ERK5, and p-CREB level normalized to CREB. Data were expressed as mean ± SEM. ***P < 0.001, compared with one hour before intrathecal injection of ephrinB2-Fc; n=8 in each group.*

3. Knockdown of ERK5 inhibited ephrinB2-Fc-induced CREB activation and hyperalgesia.

For knockdown of spinal ERK5, we designed 3 LV-siRNAs targeting at ERK5. Western blotting showed that compared to the vehicle group, LV-siERK5 1 was the most effective in inhibiting spinal ERK5 expression on day 9 after consecutive injection (Fig. 3A and 3B). In the present study, we injected Fc or ephrinB2-Fc intrathecally in rats on day 9 after LV-siERK5 1 or LV-NC consecutive injection, and measured the expression of ERK5, p-CREB, and CREB in rat L4-5 SC at 4 hours after injection by western blotting. We found that knockdown of ERK5 had no effect on the expression or activation of spinal CREB in rats injected with Fc (Fig. 3C and 3D). However, the enhanced spinal p-CREB expression after ephrinB2-Fc injection was significantly reduced by pretreatment with LV-siERK5 1, indicating that knockdown of ERK5 could inhibit the activation of CREB caused by ephrinB2-Fc (Fig. 3C and 3D). We also measured the thermal and mechanical pain threshold of the rats at different time points after Fc or ephrinB2-Fc injection. The results showed that thermal and mechanical hyperalgesia caused by ephrinB2-Fc were significantly inhibited by pretreatment with LV-siERK5 1, and the inhibition lasted for more than 48 hours after ephrinB2-Fc injection (Fig. 3E and 3F). Thus, knockdown of ERK5 could inhibit the activation of CREB and attenuate the hyperalgesia induced by ephrinB2-Fc. This result further verifies that ERK5/CREB pathway plays an important role in ephrinB-EphB signaling induced hyperalgesia.

4. Blocking EphB receptors prevented CCI-induced thermal and mechanical hyperalgesia, and spinal ERK5 and CREB activation.

CCI is one of the neuropathic pain models and could produce consecutive thermal and mechanical hyperalgesia in rats. Some studies have confirmed that CCI could induce ERK5 and CREB activation in the spinal cord (26,27). In the present study, we used EphB1-Fc (an antagonist of EphB receptor) to further examine the effect of ephrinB-EphB signaling on hyperalgesia and the activation of ERK5/CREB pathway in CCI rats. We

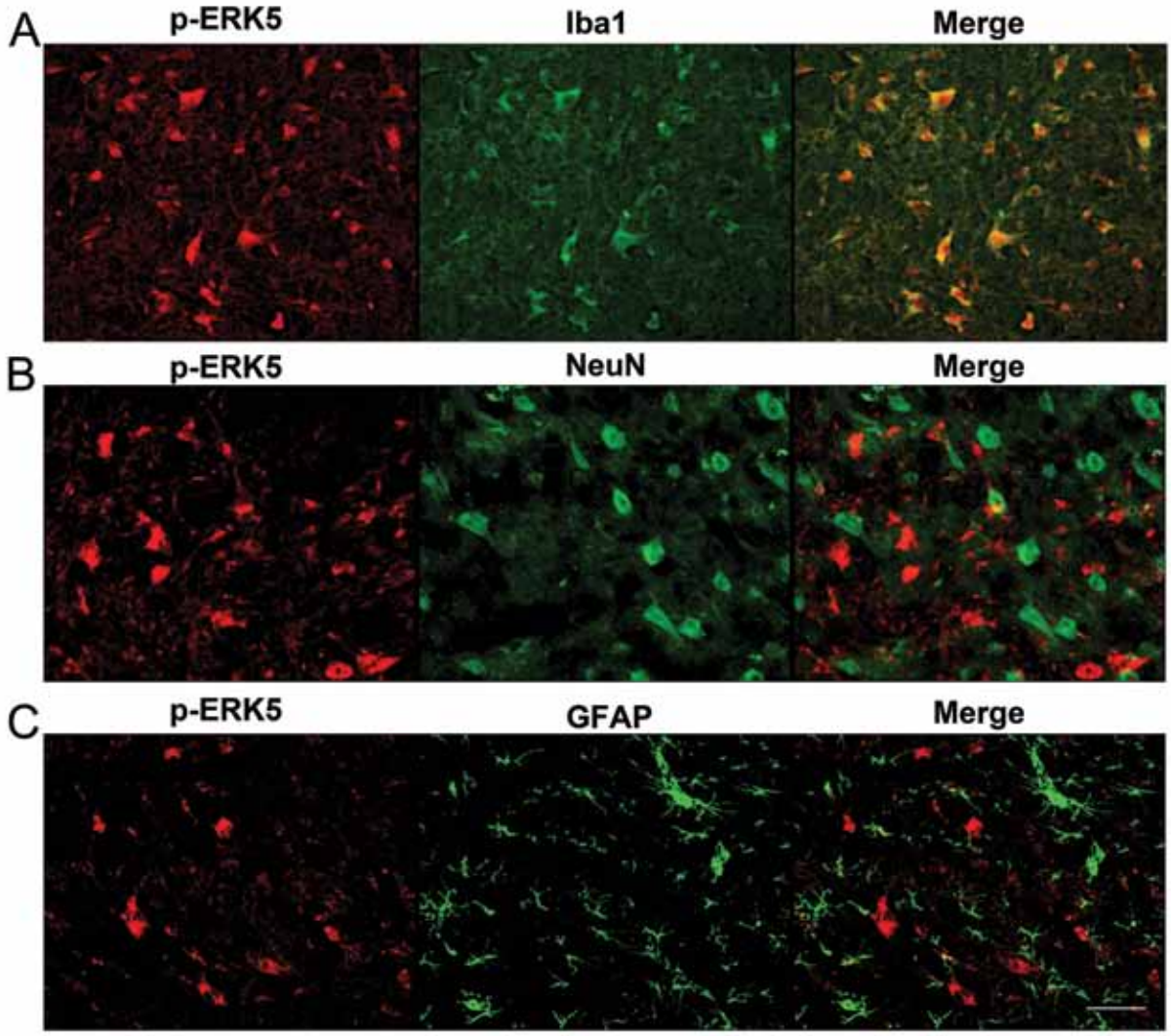

Fig. 2. *EphrinB2-Fc induced ERK5 activation in microglial cells, but not in neurons or astrocytes. (A,B,C) Immunohistochemical colocalization of p-ERK5 with Iba1, NeuN, GFAP respectively. P-ERK5 (red signals) did not colocalize with the green signals which stand for NeuN (neuron marker) or GFAP (astrocyte marker). Instead, p-ERK5 colocalized with Iba1 which is a microglia marker. Scale bar= 50 μm.*

arranged 2 parts for the experiment. Firstly, in CCI rats, intrathecal injection of EphB1-Fc was performed repeatedly in the early phase (10μg daily for 3 continuous days, starting at one hour before surgery). As shown in Fig. 4A and 4B, pretreatment with EphB1-Fc delayed thermal and mechanical hyperalgesia produced by CCI for over 14 days. We removed the L4-5 spinal cord segments from rats on day 7 after surgery, and western blotting analysis showed that pretreatment with EphB1-Fc also suppressed the CCI-induced upregulation of spinal p-ERK5 and p-CREB level (Fig. 4C and 4D). Secondly, in CCI rats, intrathecal injection of EphB1-Fc was performed repeatedly in the late phase (10μg daily for 3 continuous days, starting from day 7 after surgery). Figure 5A and 5B show the effect of EphB1-Fc post-treatment on behavioral results in CCI rats — -thermal and mechanical hyperalgesia were inhibited for about 7 days or more. We removed the L4-5 spinal cord segments from rats on day 11 after surgery (day 2 after the last administration of EphB1-Fc); the CCI-induced

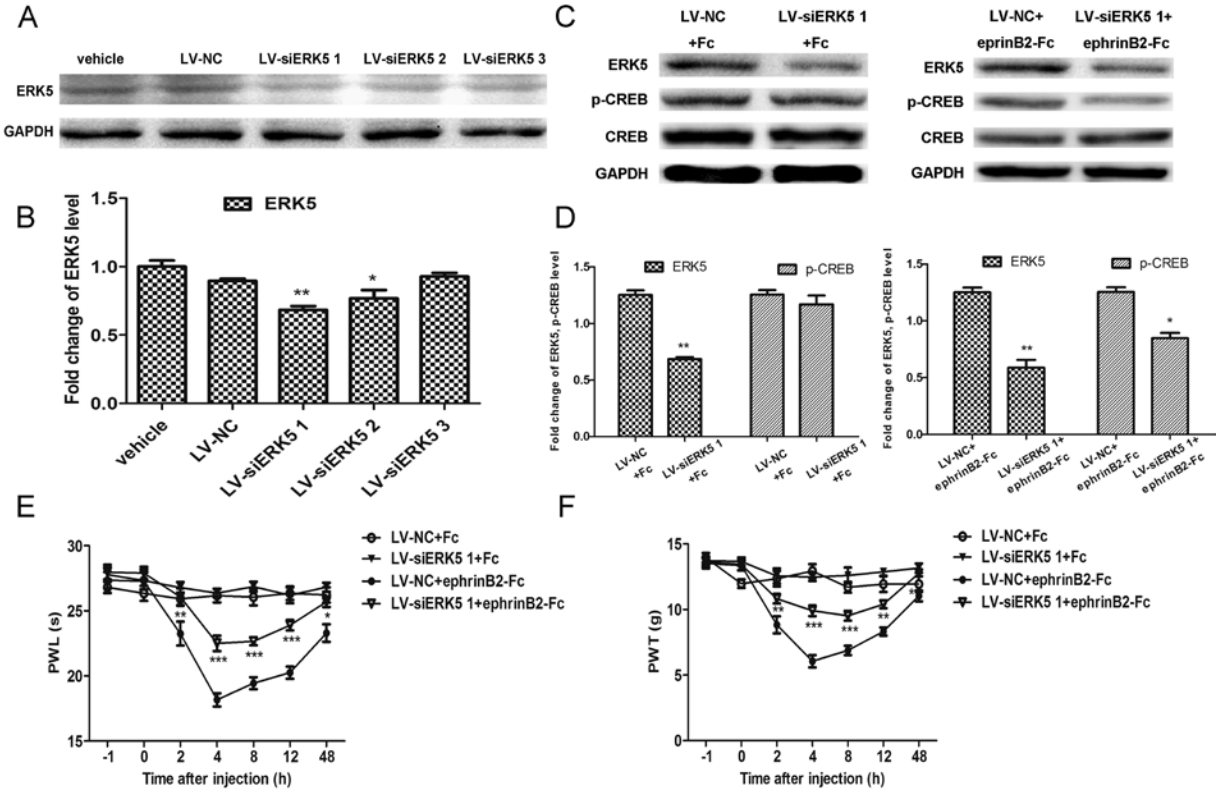

Fig. 3. *Knockdown of ERK5 inhibited ephrinB2-Fc-induced CREB activation and hyperalgesia. (A): The effect of each LV-siERK5 on the expression of ERK5 protein levels in the spinal cord. Vehicle, LV-NC, LV-siERK5 1, LV-siERK5 2, LV-siERK5 3 (10μl i.t. daily for 3 consecutive days) were administered to different groups of rats (n=6). The spinal cords were collected on day 9 after consecutive intrathecal injection. (B): Fold change for the density of ERK5 normalized to GAPDH, as shown in Figure 3A. Data were expressed as mean ± SEM. *P < 0.05, **P < 0.01, compared with vehicle group; n=6 in each group. (C): The effect of ERK5 knockdown on the expression of ERK5, p-CREB and CREB protein levels at 4h after Fc (5μg i.t.) or ephrinB2-Fc (5μg i.t.) injection. (D): The fold change for the density of ERK5 level normalized to GAPDH, and p-CREB level normalized to CREB. Data were expressed as mean ± SEM. *P < 0.05, ** P < 0.01, compared with "LV-NC +Fc" group and "LV-NC+ephrinB2-Fc" group respectively. (E,F): Knockdown of ERK5 by LV-siERK5 1 attenuated thermal and mechanical hyperalgesia induced by ephrinB2-Fc (5μg i.t.). Data were expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, compared with "LV-NC +ephrinB2-Fc" group; n=8 in each group.*

upregulation of spinal p-ERK5 and p-CREB level was also suppressed by post-treatment with EphB1-Fc (Fig. 5C and 5D).

Discussion

This study revealed the important role of ERK5/CREB pathway in ephrinB-EphB signaling induced hyperalgesia. We demonstrated the following findings: activating EphB receptors could induce thermal and mechanical hyperalgesia, along with spinal ERK5 and CREB activation; the activated ERK5 was mainly in microglia; blocking ERK5 expression could inhibit ephrinB2-Fc-induced CREB activation and hyperalgesia; the CCI-induced hyperalgesia and the activation of spinal ERK5 and CREB could be reduced by blocking EphB receptors.

EphBs and ephrinBs are bidirectional signaling; they play crucial functional roles in early segmentation and morphogenesis, vascular development in embryogenesis, and the development of the nervous system (28-30). Later in adulthood, the main roles of ephrinB-EphB signaling turn into regulation of pain threshold, epileptogenesis, neuronal reorganization, and modulation of activity-dependent synaptic plasticity in the

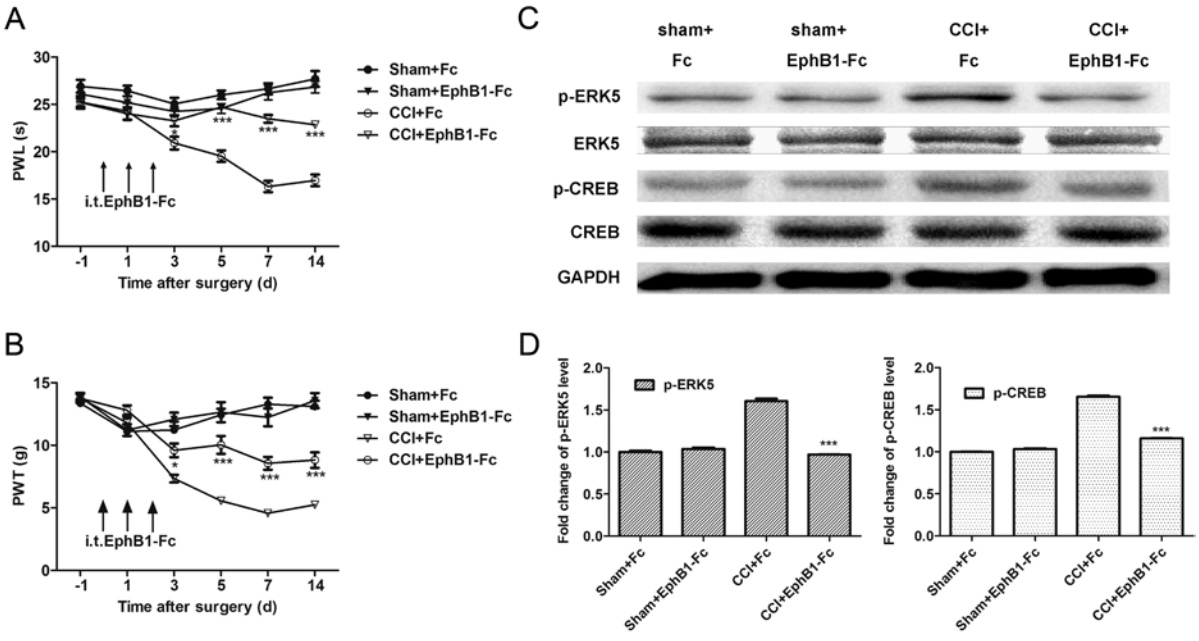

Fig. 4. *Pretreatment with EphB1-Fc prevented CCI-induced thermal and mechanical hyperalgesia, and spinal ERK5 and CREB activation. (A,B): Pretreatment with EphB1-Fc (10μg daily for 3 continuous days, starting at one hour before surgery) inhibited thermal and mechanical hyperalgesia produced by CCI. (C): The effect of pre-treatment with EphB1-Fc on CCI-induced upregulation of spinal p-ERK5 and p-CREB level. The spinal cords were collected on day 7 after surgery. (D): The fold change for the density of p-ERK5 level normalized to ERK5, and p-CREB normalized to CREB, as shown in Figure 4C. Data were all expressed as mean ± SEM. *P < 0.05, ***P < 0.001, compared with "CCI+Fc" group (Fc 10μg i.t.); n=8 in each group.*

development of chronic pain (31,32). EphrinB-EphB signaling can contribute to the formation of sensory abnormalities associated with chronic pain states, and some studies have confirmed that using siRNA targeting ephrinB2 could attenuate hyperalgesia induced by peripheral inflammation or nerve injury (31,33). Peripheral and central sensitization is the underlying mechanism of the formation of hyperalgesia, allodynia, and spontaneous pain, which are considered to be the main characteristics of chronic pain. EphrinB-EphB signaling plays important roles in the pathological process of inflammatory pain, neuropathic pain, and bone cancer pain by contributing to the establishment of central sensitization, which is an activity-dependent functional neuron plasticity (34,35). Both ephrinBs and EphB receptors could positively modulate the activity of N-methyl-Daspartate (NMDA) receptor. Previous researches have largely demonstrated the vital roles of NMDA receptor in the induction of central sensitization (32,36). It has been reported that ephrinB-EphB interactions are involved in the forming process of synaptic plasticity via an NMDA-dependent mechanism in the spinal cord: EphB receptors promote the phosphorylation of NMDA receptors' NR2B subunit and amplify the activation of NMDA receptors, the process of which is mediated by Src non-receptor tyrosine kinases family (37, 38). NMDA receptor is a type of ionotropic glutamate receptors, and one important role of the activated NMDA receptor is mediating calcium influx (39). Interactions between ephrinB-EphB can enhance the NMDA receptor-mediated Ca^{2+} influx, then trigger the downstream intracellular signaling and promote the program of some particular gene expression, and contribute to the sustained neuron hyperexcitability (38,40,41). Song et al (42) have confirmed that in neuropathic pain, activation of EphB receptors could contribute to long-term potentiation (LTP), a form of NMDA-dependent synaptic plasticity, between C afferent fibers and spinal dorsal horn neurons. Given the important roles of ephrinB-EphB signaling in the generation and maintenance of chronic pain, researchers have always focused on exploring the upstream and downstream signal pathway

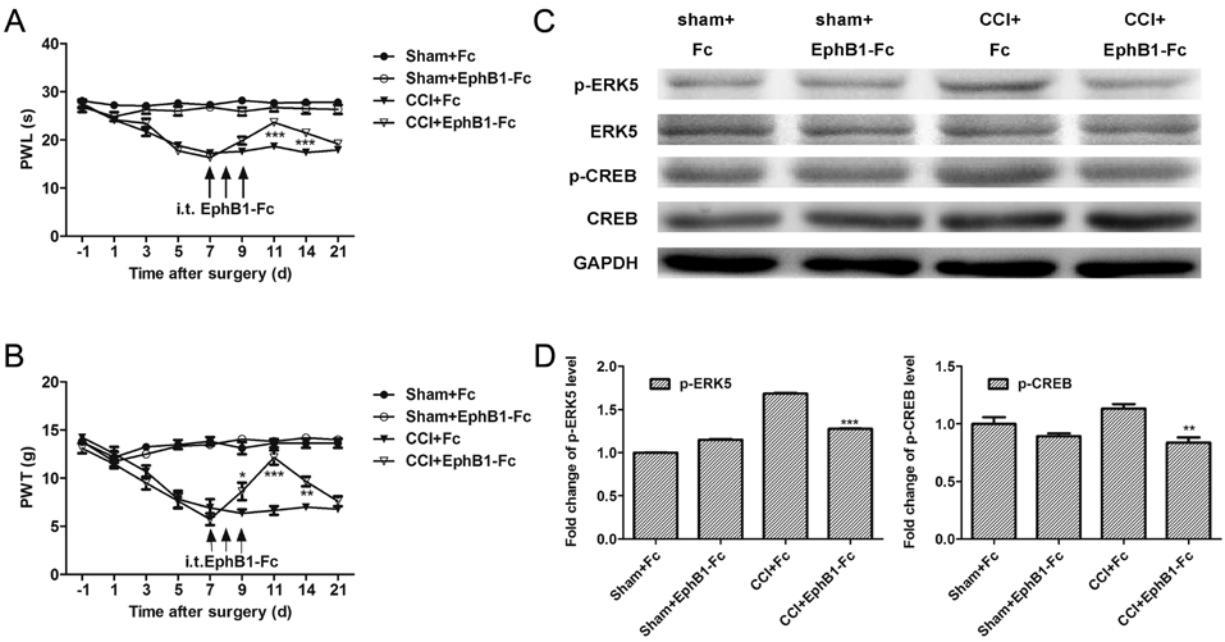

Fig. 5. *Posttreatment with EphB1-Fc prevented CCI-induced thermal and mechanical hyperalgesia, and spinal ERK5 and CREB activation. (A,B): Posttreatment with EphB1-Fc (10μg daily for 3 continuous days, starting on day 7 after surgery) inhibited thermal and mechanical hyperalgesia produced by CCI. (C): The effect of posttreatment with EphB1-Fc on CCI-induced upregulation of spinal p-ERK5 and p-CREB level. The spinal cords were collected on day 11 after surgery. (D): The fold change for the density of p-ERK5 level normalized to ERK5, and p-CREB level normalized to CREB, as shown in Figure 5C. Data were all expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, compared with "CCI+Fc" group (Fc 10μg i.t.); n=8 in each group.*

of ephrinBs and EphB receptors. The researchers have made great progress in recent years. For example, Cao et al (11,12) have demonstrated that some MAPKs such as ERK1/2, p38, and JNK, are involved in the ephrinB-EphB signaling-induced hyperalgesia. Our previous studies have consecutively proved that phosphatidy linositol 3-kinase (PI3K), protein kinase A (PKA), and protein kinase Cγ (PKCγ) act as the downstream factors of ephrinB-EphB signaling in modulating the spinal nociceptive information (7,43-45). In the present study, we hypothesized that ERK5/CREB pathway may act as the downstream signal pathway of ephrinB-EphB signaling in modulating pain transduction. We have confirmed this hypothesis.

ERK5 is a member of MAPK family. It is well established that MAPKs could mediate the transduction of pain signals and contribute to central sensitization in various kinds of pathological pain (46). As mentioned above, some members of MAPKs take part in mediating ephrinB-EphB signaling-induced pain hypersensitivity (13). In recent years, increasing studies have reported that ERK5 activation in the dorsal root ganglion and the spinal cord are involved in modulating nociceptive information in inflammatory or neuropathic pain (26,47). Activation of ERK5 can be mediated by NMDA receptors and the subsequent associated intracellular signal transduction cascades (39). In the transduction of nociceptive information, activated NMDA receptors can trigger an increase of intracellular Ca^{2+} concentration and activate the ERK5 signal pathway; activated ERK5 then transmit signals to the nucleus by phosphorylating several nuclear transcription factors and adjust the downstream gene expression (27). CREB is one of the nuclear transcription factors, which are the downstream targets of ERK5 (17). P-ERK5 phosphorylates the transcription factor CREB through the activation of p90 ribosomal S6 kinase (RSK); p-CREB then binds to the cAMP-response element sites (CRE) in the promoter regions of the DNA and initiates the transcription of some pain-related genes including c-fos, zif268, COX-2, NK-1, dynorphin, CGRP, and BDNF (48,49). The CREB-dependent gene expression has been suggested to

regulate synaptic plasticity and contribute to central sensitization during persistent pain (19). In the present study, our results revealed that ephrinB-EphB signaling can induce hyperalgesia through ERK5/CREB pathway in rats. Intrathecal injection of ephrinB2-Fc in rats evokes hyperalgesia, along with activation of ERK5 and CREB in the spinal cord. Knockdown of spinal ERK5 inhibits ephrinB2-Fc-induced CREB activation and hyperalgesia. Blocking EphB receptors prevents CCI-induced neuropathic pain and inhibits spinal ERK5/CREB activation. These present findings all support the conclusion that ERK5/CREB pathway plays an important role in the transduction of nociceptive information associated with ephrinB-EphB signaling.

What is the potential mechanism that underlies the relationship between ephrinB-EphB signaling and ERK5/CREB pathway? Given that NMDA receptor is a downstream target of EphB receptor and an upstream regulator of ERK5/CREB pathway, it is reasonable to make an assumption that NMDA receptor and its subsequent Ca^2+ influx may modulate the activation of ERK5 and CREB in the ephrinB-EphB signaling-induced hyperalgesia. Meanwhile, it is also possible that proinflammatory cytokines may mediate the process. It has been reported that ephrinB-EphB signaling contributes to bone cancer pain via activating glial cells and increasing the release of proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (50). Proinflammatory cytokines are known to be involved in the formation of behavioral hypersensitivity and the induction of central sensitization in chronic pain states (51). Furthermore, ERK5 activation is mainly in microglias, which have been proved to contribute to the development of neural plasticity after nerve injury or inflammation via accelerating the production of proinflammatory cytokines including IL-1, IL-6, and TNF-α (52,53). Those proinflammatory cytokines may mediate the activation of ERK5 in microglias. Thus, it is also probable to suppose that proinflammatory cytokines may modulate the activation of ERK5 in the spinal cord in ephrinB-EphB signaling-induced hyperalgesia. The specific mechanisms of ERK5/CREB pathway activation induced by ephrinB-EphB signaling still need more research done, to study in the future.

In conclusion, our present findings confirm the role of ERK5/CREB pathway involved in ephrinB-EphB signaling induced hyperalgesia. Together with our previous researches, we provided further mechanisms for ephrinB-EphB system in pathological pain signal transduction. Our findings may provide new insights into the molecular mechanisms underlying ephrinBs/EphBs signaling in modulating neuropathic pain. It suggests that ephrinB-EphB signaling and its downstream ERK5/CREB pathway may be potential targets for blocking pain signal transduction. Thus, this finding will help us exploit new therapeutic opportunities for clinical analgesia in the future.

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