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EphrinBs/EphBs Signaling Is Involved in Modulation of Spinal Nociceptive Processing through a Mitogen-activated Protein Kinases-dependent Mechanism

Jia-Ping Ruan, M.D.,* Hong-Xing Zhang, M.D.,† Xian-Fu Lu, M.D.,‡ Yue-Peng Liu, M.D.,§ Jun-Li Cao, M.D., Ph.D.‖

ABSTRACT

Background: Our previous studies have demonstrated that EphBs receptors and ephrinBs ligands were involved in modulation of spinal nociceptive information. However, the downstream mechanisms that control this process are not well understood. The aim of this study was to further investigate whether mitogen-activated protein kinases (MAPKs), as the downstream effectors, participate in modulation of spinal nociceptive information related to ephrinBs/EphBs.

Methods: Thermal hyperalgesia and mechanical allodynia were measured using radiant heat and von Frey filaments test. Immunofluorescence staining was used to detect the expression of p-MAPKs and of p-MAPKs/neuronal nuclei, or p-MAPKs/glial fibrillary acidic protein double label. C-Fos expression was determined by immunohistochemistry. The expression of p-MAPKs was also determined by Western blot assay.

Results: Intrathecal injection of ephrinB1-Fc produced a dose- and time-dependent thermal and mechanical hyperalgesia, accompanied by the increase of spinal p-MAPKs and c-Fos expression. Immunofluorescence staining revealed that p-MAPKs colocalized with the neuronal marker (neuronal nuclei) and the astrocyte marker (glial fibrillary acidic protein). Inhibition of MAPKs prevented and reversed pain behaviors and the increase of spinal c-Fos expression induced by intrathecal injection of ephrinB1-Fc. Inhibition of EphBs receptors by intrathecal injection of EphB1-Fc reduced formalin-induced inflammation and chronic constrictive injury-induced neuropathic pain behaviors accompanied by decreased expression of spinal p-MAPKs and c-Fos protein. Furthermore, pretreatment with MK-801, an *N*-methyl-D-aspartate receptor antagonist, prevented behavioral hyperalgesia and activation of spinal MAPKs induced by intrathecal injection of ephrinB1-Fc.

Conclusions: These results demonstrated that activation of MAPKs contributed to modulation of spinal nociceptive information related to ephrinBs/EphBs.

What We Already Know about This Topic

- Eph receptor tyrosine kinases and their ligands, ephrins in sensory neurons may play a role in hypersensitivity after nerve injury
- In the periphery, Eph receptors stimulate protein kinase (MAPK) activity

What This Article Tells Us That Is New

- In mice, intrathecal injection of ephrins stimulated MAPK activity in spinal cord neurons and astrocytes and caused hypersensitivity
- Blockade of MAPK activity prevented hypersensitivity from ephrins and blockade of Eph receptors in the spinal cord reduced hypersensitivity and MAPK activity in a model of neuropathic pain

EPH receptor tyrosine kinases and their membrane-bound ligands, ephrins, are involved in diverse aspects of development, such as tissue patterning, angiogenesis, axon guidance, and synapse formation.[1–4] Recent advances indicate that Eph receptors and ephrin ligands are present in the adult brain and peripheral tissue and play a critical role in modulating multiple aspects of physiology and pathophysiology (*e.g.*, activity-dependent synaptic plasticity, regulation of pain threshold, epileptogenesis, inflammation response, and excitotoxic neuronal death).[5–9] Interestingly, several Eph receptors and ephrin ligands are also expressed in the adult rat spinal cord and the dorsal root ganglion.[10–12] Bundesen *et al.*[12] reported that EphB2 receptor was present in the laminae I–III of the dorsal horn and on small- and medium-sized dorsal root ganglion neurons but not on large-diameter neurons, two important sites for modula-

* Graduate Student, Jiangsu Key Laboratory of Anesthesiology, Xuzhou Medical College, Xuzhou, China, and Staff Anesthesiologist, Department of Anesthesiology, Jiangsu Provincial Hospital of Integrated Chinese Medicine and Western Medicine, Nanjing, China. † Graduate Student, § Research Associate, Jiangsu Key Laboratory of Anesthesiology, Xuzhou Medical College. ‡ Ph.D. Student, China Medical University, Shenyang, China. ‖ Associated Professor, Department of Anesthesiology, Affiliated Hospital of Xuzhou Medical College, Jiangsu Institute of Anesthesiology, Jiangsu Key Laboratory of Anesthesiology.

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Address correspondence to Dr. Cao: Department of Anesthesiology, Affiliated Hospital of Xuzhou Medical College, 99 Huaihai West Road, Xuzhou City, Jiangsu Province 221002, China. caojl0310@yahoo.com.cn.

tion of nociceptive information. The interactions of ephrinB–EphB modulate synaptic efficacy in the spinal cord, contributing to sensory abnormalities in persistent pain states in an *N*-methyl-D-aspartate (NMDA) receptor-dependent manner.[13,14] Recently, our and other studies also demonstrated that activation of spinal ephrinBs/EphBs system played a critical role in the development and maintenance of chronic pain after peripheral nerve injury.[15–17] These studies indicated that Ephrin/Eph system may be involved in physiologic and pathologic pain modulation in spinal cord level. However, the downstream mechanisms that control this process are not well understood.

It is well established that the mitogen-activated protein kinases (MAPKs) activation is involved in the modulation of nociceptive information and peripheral and central sensitization produced by intense noxious stimuli.[18–23] Several lines of evidence have shown that regulation of MAPKs is associated with EphBs receptors activation.[24–27] Our recent study showed that MAPKs signaling pathway mediated hyperalgesia induced by the activation of peripheral ephrinBs/EphBs system.[28] Thus, it is possible that MAPKs, as the downstream effectors, participate in modulation of nociceptive information related to ephrinBs/EphBs signaling in the spinal cord level. We provide strong evidence to support this hypothesis.

Materials and Methods

Animals

Adult male Kunming mice (20–25 g) were used in these studies. Mice were housed under a 12 h/12 h light–dark cycle regimen, with free access to food and water. The animals were provided by Experimental Animal Center of Xuzhou Medical College. All experimental protocols were approved by the Animal Care and Use Committee of Xuzhou Medical College (Xuzhou, Jiangsu Province, China) and were in accordance with the Declaration of the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (publication no. 80–23, revised 1996).

Drug Application

SB203580, a p38 kinase inhibitor; U0126, the mitogen-activated protein or extracellular signal-regulated kinase inhibitor; and SP600125, a Jun N-terminal kinases (JNK) inhibitor were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). All MAPKs inhibitors were dissolved in 1% dimethyl sulfoxide (DMSO). EphrinB1-Fc and EphB1-Fc were purchased from R&D Systems Inc. (Minneapolis, MN) and are dissolved in saline. All doses of drugs are based on the results of preliminary experiments. The dose of each drug and time points of treatment were presented in the parts of figure legends.

The method described by Hylden and Wilcox[29] was used for an intrathecal injection of drugs. In brief, a 28-gauge stainless steel needle attached to a 25-μl Hamilton microsyringe was inserted between the L5 and L6 vertebrae in conscious mice. A sudden slight flick of the tail indicated entry into the subarachnoid space. A volume of 5 μl of drug solution or physiologic saline was injected over a 30-s period into the subarachnoid space, and the injection cannula was left in place for a further 15 s. Motor function was evaluated by observation of placing or stepping reflexes and righting reflexes at 2 min before nociceptive test. Animals with signs of motor dysfunction were excluded from the experiments.

Chronic Constrictive Injury Model

Chronic constrictive injury (CCI) model was performed following the method of Bennett and Xie.[30] In brief, mice were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal injection). Left sciatic nerve was exposed at midthigh level through a small incision, and a unilateral constriction injury just proximal to the trifurcation was performed with three loose ligatures using a 5-0 silk thread (spaced at a 1-mm interval). In sham-operated animals, the nerve was exposed but not ligated. The incision was closed in layers, and the wound was treated with antibiotics.

Formalin Test

The procedure used was essentially the same as that reported by Hunskaar and Hole.[31] Approximately 30 min before testing, mice were individually placed in perspex observation chambers (10 × 20 × 15 cm) for adaptation. Then, the animals were taken out of the chamber, and 10 μl of 1% formalin in 0.9% saline was injected subcutaneously into the dorsal surface of the right hind paw with a 25-μl Hamilton syringe with a 28-gauge needle. Immediately after formalin injection, each mouse was returned to the observation chamber. The amount of time spent licking and biting the injected paw and the number of flinching paw were measured from 0 to 5 min (the first phase) and from 10 to 40 min (the second phase) after formalin injection and was considered as indicative of nociception.

Measurement of Thermal Hyperalgesia

Thermal hyperalgesia was measured using the paw-withdrawal latency (PWL) according to the method described by Hargreaves *et al.*[32] In brief, mice were placed in clear plastic chambers (7 × 9 × 11 cm) and allowed to acclimatize to the environment for 1 h before testing. The radiant heat was directed to the plantar surface of each hind paw that was flushed against the glass or site of injection of solution through the glass plate. 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 PWL. The radiant heat intensity was adjusted to obtain basal PWL of 12–15 s. An automatic 20-s cutoff was used to prevent tissue damage. Thermal stimuli were delivered three times to each hind paw at 5-min intervals.

In CCI model, withdrawal latencies were measured in the same animal on both the ipsilateral (ligated) and the contralateral (unligated) paw. Results were expressed as the difference scores of PWL (s) = contralateral latency − ipsilateral latency.

Measurement of Mechanical Allodynia

Mechanical allodynia was assessed by using von Frey filaments (North Coast Medical, Inc., San Jose, CA), starting with 0.31 g and ending with 4.0 g filament as cutoff value. Animals were placed in individual plastic boxes (20 × 25 × 15 cm) on a metal mesh floor and allowed to acclimate for 1 h. The filaments were presented, in ascending order of strength, perpendicular to the plantar surface with sufficient force to cause slight bending against the paw and held for 6–8 s. Brisk withdrawal or paw flinching was considered as positive responses. The paw-withdrawal threshold (PWT) was determined by sequentially increasing and decreasing the stimulus strength (the "up-and-down" method), and the data were analyzed using the nonparametric method of Dixon, as described by Chaplan *et al.*[33]

In CCI model, withdrawal thresholds were measured in the same animal on both the ipsilateral (ligated) and the contralateral (unligated) paw. Results were expressed as the difference scores of PWT (g) = contralateral threshold − ipsilateral threshold. The behavioral testing was performed by an investigator blinded to the treatment.

Immunohistochemistry

Mice were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal injection) and were subjected to sternotomy followed by intracardial perfusion with 20 ml saline and 100 ml 4% ice-cold paraformaldehyde in 0.1 mol/l phosphate buffer. The spinal cord of L_{4-5} was removed, postfixed in 4% paraformaldehyde for 3 h, and subsequently allowed to equilibrate in 30% sucrose in phosphate buffer overnight at 4°C. Thirty micrometer transverse series sections were cut on a cryostat and stored in phosphate buffer. After washing in phosphate buffer saline, the tissue sections were incubated in phosphate buffer saline containing 5% normal goat serum and 0.3% Triton X-100 at room temperature for 30 min. For the Fos protein assay, the sections were incubated in primary polyclonal rabbit-anti-Fos antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 48 h. The sections were then incubated in biotinylated goat anti-rabbit (1:200) at 37°C for 1 h and in avidin–biotin–peroxidase complex (1:100; Vector Labs, Burlingame, CA) at 37°C for 2 h. Finally, the sections were treated with 0.05% diaminobenzidine for 5–10 min. Sections were rinsed in phosphate buffer saline to stop the reaction, mounted on gelatin-coated slides, air-dried, dehydrated with 70–100% alcohol, cleared with xylene, and cover slipped for microscopic examination. For analysis of the change of Fos protein expression, we examined five L_{4-5} spinal cord sections per animal, selecting the sections with the greatest number of positive neurons. For each animal, we recorded the total number of positive neurons in the bilateral spinal cord I–V and X lamina. All positive neurons were counted without considering the intensity of the staining.

For double immunofluorescence staining, the sections were incubated overnight at 4°C with a mixture of monoclonal antiphosphorylated MAPKs (p-MAPKs) antibody (anti-p-p38 from Signalway [Nanjing, China], anti-p-ERK and anti-p-c-JNK from Cell Signaling Technology [Danvers, MA], 1:200) and monoclonal neuronal-specific nuclear protein (NeuN; neuronal marker, antimouse, 1:100; Chemicon, Temecula, CA) or glial fibrillary acidic protein (astrocyte marker, anti-mouse, 1:200; Chemicon) followed by a mixture of Rhodamine Red-X goat anti-rabbit immunoglobulin G and fluorescein isothiocyanate monoclonal rat anti-mouse immunoglobulin G (1:100; Invitrogen, Carlsbad, CA) for 2 h at room temperature. Nonspecific staining was determined by excluding the primary antibodies. Sections were rinsed, mounted, and cover slipped with glycerol containing 2.5% of antifading agent 1,4-diazabicyclo[2.2.2]octane (Sigma, St. Louis, MO) and stored at −20°C in the dark. Images were captured using a laser scanning confocal microscopy (TCS SP2; Leica Microsystems Inc., Wetzlar, Germany).

Western Blot Analysis

The spinal cords of mice were quickly extracted and stored in liquid nitrogen. Tissue samples were homogenized in lysis buffer containing (in millimoles): Tris, 20.0 mM; sucrose, 250.0 mM; Na_3VO_4, 0.03 mM, $MgCl_2$, 2.0 mM, EDTA, 2.0 mM, EGTA, 2.0 mM; phenylmethylsulfonyl fluoride, 2.0 mM; dithiothreitol, 1.0 mM; protease inhibitor cocktail, 0.02% (v/v); pH 7.4. The homogenates were centrifuged at 5,000 *g* for 30 min at 4°C. The supernatant was collected, and protein concentration was measured according to the Bradford[34] method, using bovine serum albumin as standard. The protein samples were stored at −80°C.

Protein samples were dissolved in 4× sample buffer (pH 6.8): Tris–HCl, 250.0 mM; sucrose, 200.0 mM; dithiothreitol, 300.0 mM; Coomassie brilliant blue-G, 0.01%; and sodium dodecyl sulfate, 8% and denatured at 95°C for 5 min, then the equivalent amounts of proteins (30 μg) were separated by using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. In addition, the gels stained with Coomassie blue were used to confirm the equal amounts of protein loaded on each lane. The membranes were incubated overnight at 4°C with the following primary antibodies: primary polyclonal rabbit anti-p-p38 or anti-p38 antibody (1:400), primary polyclonal rabbit anti-p-ERK1/2 or anti-ERK1/2 antibody (1:400), or primary polyclonal rabbit-anti-p-JNK or anti-JNK antibody (1:400). The specificity for p-MAPKs antibodies was confirmed by loss of bands in the absence of primary antibodies. The membranes were extensively washed with Tris-buffered saline Tween 20 and incubated for 1 h with the secondary antibody conjugated with alkaline phosphatase (1:1,000) at room temperature. The immune complexes were detected by using a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate assay kit (Sigma). Western blot densitometry analysis of signal intensity was performed using Adobe Photoshop software (Adobe, San Jose, CA), and phosphorylation levels of MAPKs from densitometry were nor-

malized to total MAPKs. The blot density from control groups was set as 100%.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis between two samples was performed using Student *t* test. Statistical comparison of more than two groups was performed using one-way ANOVA followed by a Tukey *post hoc* test. The significance of any differences in thermal latency and mechanical threshold in behavior test was assessed using two-way ANOVA. "Time" was treated as "within subjects" factor and "treatment" was treated as a "between subjects" factor. The area under the pain threshold change *versus* time curve was calculated by GraphPAD Prism5 (Graph Pad Software, Inc., San Diego, CA) in some behavioral test. Statistical analyses of data were generated using GraphPAD Prism 5. All *P* values given are based on two-tailed tests. A value of *P* less than 0.05 was considered as statistically significant.

Results

Intrathecal Injection of EphrinB1-Fc Induced a Time- and Dose-dependent Hyperalgesia and Spinal Fos Expression

Previous study has shown that activation of spinal EphBs receptors by intrathecal injection of ephrinB2-Fc produced prolonged thermal hyperalgesia, but not mechanical allodynia, in the rats.[13] Our results were in partial agreement with this study. We found that intrathecal injection of ephrinB1-Fc (0.02, 0.1, and 0.5 μg in 5 μl saline), not control Fc (see experimental protocol in fig. 1A), induced a dose-dependent thermal hyperalgesia and mechanical allodynia in the mice, which can last at least up to 24 h and return to baseline level on 48 h after injection of ephrinB1-Fc ($P < 0.05$, from 0.5 to 24 h time point, ephrinB1-Fc0.1 or ephrinB1-Fc0.5 group *vs.* saline or Fc group; fig. 1B). Compared with saline and Fc groups, the calculated area under the curve (−2–48 h) in PWL and PWT tests was significantly decreased in a dose-dependent manner in ephrinB1-Fc group (fig. 1B inset). To rule out a nonspecific effect through the Fc portion, human Fc was used as a control. No significant hyperalgesia was detected after injection of human Fc. Intrathecal injection of both ephrinB1-Fc and ephrinB2-Fc can activate EphBs receptor. To date, we cannot identify which EphBs subgroup was activated dominantly by ephrinB1-Fc or ephrinB2-Fc. Various species, not various regents, used in these two experiments may be the main cause of difference in mechanical allodynia induced by intrathecal injection of ephrinBs-Fc in rats and mice.

Spinal neuronal sensitization was involved in the development and maintenance of hyperalgesia induced by the different causes. Fos protein, the product of c-fos immediate early gene, has been used as a maker for neuronal activation in the central nervous system.[35,36] There is a positive correlation between the quantity of Fos protein expression and the neuronal activation induced by nociceptive stimuli in spinal cord neurons. To further clarify the algesic effect of intrathecal injection of ephrinB1-Fc, we investigated the change of spinal Fos protein expression induced by intrathecal injection of different doses of ephrinB1-Fc (0.02, 0.1, and 0.5 μg in 5 μl saline). Intrathecal injection of ephrinB1-Fc, not saline or Fc, induced a dose-dependent increase in spinal Fos protein expression. The expression of Fos protein mainly distributed in I–V lamina and surrounding of center canal (X lamina) of spinal cord ($P < 0.0001$, ephrinB1-Fc0.1 or ephrinB1-Fc0.5 group *vs.* saline or Fc group; fig. 1C). These results further confirmed that activation of spinal ephrinBs/EphBs system can sensitize the spinal neurons and induce pain behaviors in naïve mice.

Intrathecal Injection of EphrinB1-Fc Induced a Time- and Dose-dependent Activation of Spinal MAPKs

Some studies have shown that spinal MAPKs activation mediated inflammatory or injury-induced spinal sensitization and hyperalgesia.[18–23] Furthermore, studies *in vitro* and *in vivo* also have indicated that ephrinBs/EphBs signaling is involved in the regulation of MAPKs activity.[24–27,37,38] Thus, we ask whether activation of spinal MAPKs pathway contributes to ephrinB1-Fc-induced pain behaviors. Phosphorylation level of MAPKs correlates with MAPKs activation and is used routinely as an indicator of MAPKs activation.[18] In the current studies, MAPKs activation was evaluated by detecting the expression of p-MAPKs by Western blot analysis in mice. We first analyzed the time course of expression of spinal p-MAPKs, including p-p38, p-ERK, and p-JNK, after intrathecal injection of ephrinB1-Fc (0.5 μg). A rapid ERK activation was detected at 10 min, and the significant increase in p-p38 and p-JNK expression was detected at 30 min after intrathecal injection of ephrinB1-Fc. Activation of three MAPKs lasted at least up to 2 h and returned to baseline levels on 8 h after intrathecal injection of ephrinB1-Fc (p-p38: $P < 0.01$ at 0.5 h, $P < 0.05$ at 2 h; p-ERK: $P < 0.01$ at 0.17 h, $P < 0.05$ at 0.5 and 2 h; p-JNK: $P < 0.05$ at 0.5 and 2 h, *vs.* 0-h; fig. 2A). To further determine ephrinB1-induced MAPKs activation, we picked up 30-min time point to perform dose–response experiments. Intrathecal injection of ephrinB1-Fc (0.02, 0.1, and 0.5 μg), not Fc, induced a dose-dependent increase in spinal p-MAPKs expression ($P < 0.05$ or $P < 0.01$, ephrinB1-Fc0.1 or ephrinB1-Fc0.5 group *vs.* Fc group; fig. 2B).

Cellular Localization of Spinal Activated MAPKs

Activated MAPKs in both neurons and spinal glial cells were involved in spinal nociceptive information modulation.[23,39–42] Previous studies have shown that ephrinBs or EphBs is expressed in astrocytes of the adult spinal cord.[12] To identify the cell types that expressed spinal p-MAPKs

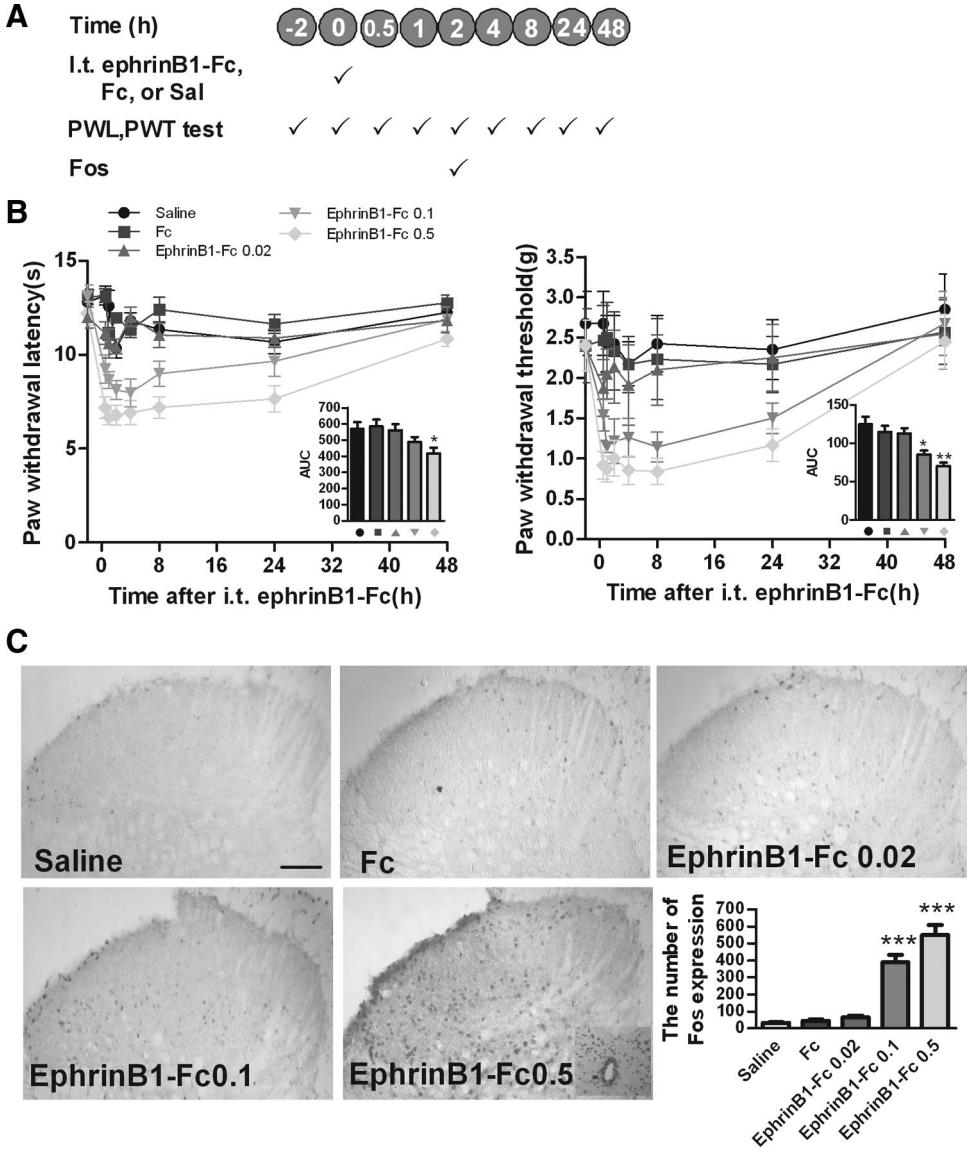

Fig. 1. Intrathecal (i.t.) injection of ephrinB1-Fc produced a dose- and time-dependent hyperalgesia and spinal Fos protein expression. (*A*) Schematic illustration of experimental protocol. (*B*) Intrathecal injection of 0.1 or 0.5 μg ephrinB1-Fc, not 0.02 μg ephrinB1-Fc or 0.5 μg control Fc, produced thermal hyperalgesia and mechanical allodynia. $P < 0.05$, from 0.5 to 24 h time point, ephrinB1-Fc0.1 or ephrinB1-Fc0.5 group *vs.* saline or Fc group. Inset figures showed that the calculated area under the curve (AUC) (−2–48 h) in paw-withdrawal latency (PWL) and paw-withdrawal threshold (PWT) test was significantly decreased in a dose-dependent manner in ephrinB1-Fc group. * $P < 0.05$, ** $P < 0.01$ compared with saline or Fc group. n = 8 mice in each group. (*C*) Representative immunohistochemical staining and the quantitative data of Fos expression in the spinal cord of mice. Intrathecal injection of ephrinB1-Fc (0.02, 0.1, and 0.5 μg), not control Fc (0.5 μg), induced a dose-dependent increase in spinal Fos protein expression. A representative staining for Fos protein expression in surrounding of center canal of the spinal cord was shown in ephrinB1-Fc0.5 group. *** $P < 0.0001$ compared with saline group, n = 4 mice in saline or Fc group, n = 6 mice in ephrinB1-Fc0.02, 0.1, or 0.5 group. Scale bar = 100 μm.

after intrathecal injection of ephrinB1-Fc, double immunofluorescence staining of p-MAPKs was performed with cell-specific markers: NeuN for neurons, glial fibrillary acidic protein for astrocytes at 30 min after intrathecal injection of ephrinB1-Fc (0.5 μg; fig. 3). We found that the activated MAPKs by intrathecal injection of ephrinB1-Fc were primarily expressed in spinal cord dorsal horn (fig. 3A). Furthermore, MAPKs in dorsal root ganglion also were activated by intrathecal injection of ephrinB1-Fc (fig. 3C). All three phosphorylated MAPKs colocalized with the neuronal marker (NeuN; fig. 3B) and p-p38 and p-JNK, not p-ERK, with the astrocyte marker (glial fibrillary acidic protein) in the spinal cord (fig. 3D).

Inhibition of Spinal MAPKs Prevented and Reversed Pain Behaviors Induced by Intrathecal Injection of EphrinB1-Fc

The current data have indicated that intrathecal injection of ephrinB1-Fc induced thermal hyperalgesia and mechanical allodynia, which was associated with activation of spinal MAPKs. Therefore, inhibition of activation of

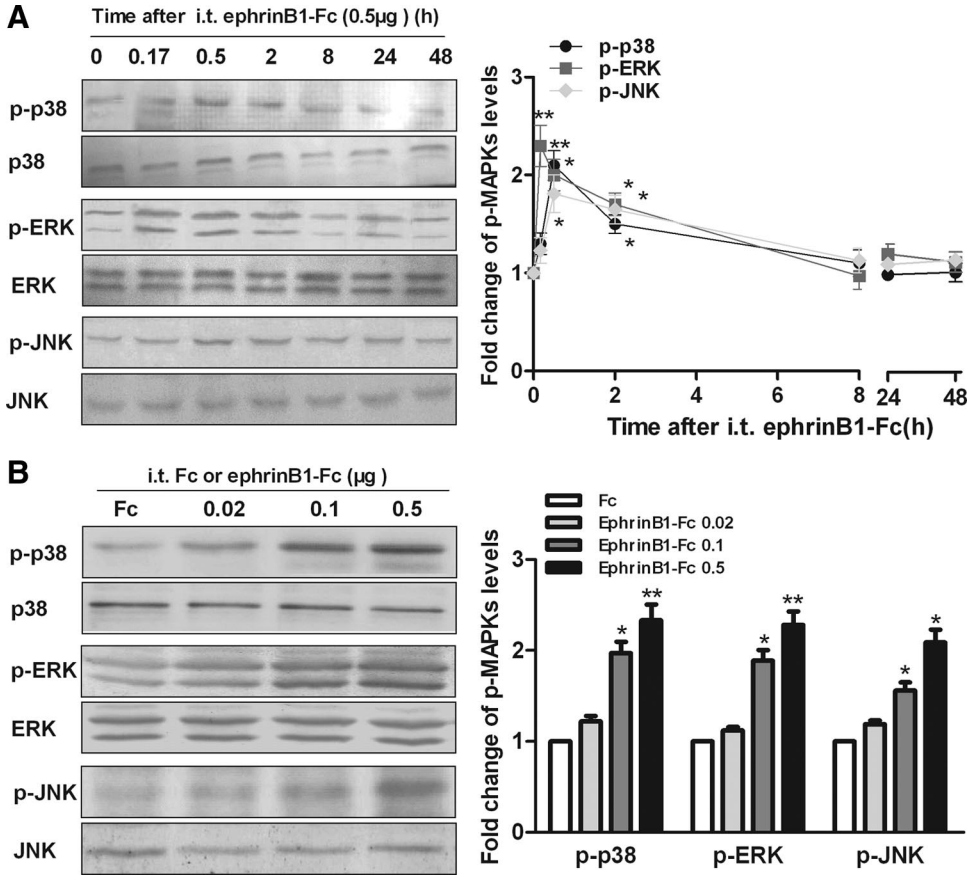

Fig. 2. Intrathecal (i.t.) injection of ephrinB1-Fc induced a dose- and time-dependent activation of spinal mitogen-activated protein kinases (MAPKs). (*A*) The representative bands for the expression of spinal p-p38, p-ERK, and p-JNK at different time points after injection of ephrinB1-Fc (0.5 μg) and the quantitative data for the expression of each phosphorylated MAPKs (p-MAPKs). The fold change for the density of each p-MAPKs normalized to total MAPKs for each sample. The fold change of p-MAPKs levels in 0 time point group was set at 1 for quantifications. Compared with 0 h time point, p-p38: ** $P < 0.01$ at 0.5 h, * $P < 0.05$ at 2 h; p-ERK: ** $P < 0.01$ at 0.17 h, * $P < 0.05$ at 0.5 and 2 h; p-JNK: * $P < 0.05$ at 0.5 and 2 h, n = 4 mice in each group. (*B*) The representative bands for the expression of spinal p-MAPKs after injection of various dose of ephrinB1-Fc (0.02, 0.1, and 0.5 μg) or control Fc (0.5 μg) and the quantitative data for the expression of each p-MAPKs. The fold change for the density of each p-MAPKs normalized to total MAPKs for each sample. The fold change of p-MAPKs levels in Fc group was set at 1 for quantifications. * $P < 0.05$, ** $P < 0.01$ compared with Fc group, n = 4 mice in each group.

spinal MAPKs will alleviate pain behaviors in theory. As shown in figure 4, pretreatment with a p38 MAPK inhibitor SB203580 (SB), an MEK inhibitor U0126 (U) or SP600125 (SP), a JNK MAPK inhibitor (0.02, 0.1, and 0.5 μg in 1% DMSO for each inhibitor) at 30 min before ephrinB1-Fc injection (see experimental protocol in fig. 4A) dose dependently prevented thermal hyperalgesia (fig. 4C) and mechanical allodynia (fig. 4D) induced by intrathecal injection of ephrinB1-Fc (0.5 μg). Compared with DMSO-ephrinB1-Fc group, the calculated area under the curve (−2–48 h) in PWL and PWT tests was significantly increased in a dose-dependent manner in SB-ephrinB1-Fc, U-ephrinB1-Fc, and SP-ephrinB1-Fc groups (fig. 4C and D inset). It suggested that activation of MAPKs was involved in the initiation of pain behaviors by ephrinB1-Fc. Then, we asked whether spinal MAPKs pathway also participate in its maintenance process. To address this question, SB, U, or SP (0.5 μg for each inhibitor) was administrated at 30 min after intrathecal injection of ephrinB1-Fc (0.5 μg; see experimental protocol in fig. 4B). We found that all three MAPKs inhibitors reversed the established thermal hyperalgesia and mechanical allodynia by intrathecal injection of ephrinB1-Fc. This effect lasted at least up to 8 h after injection of MAPKs inhibitors (fig. 4E). Compared with ephrinB1-Fc-DMSO group, the calculated area under the curve (−2–8 h) in PWL and PWT tests was significantly increased in ephrinB1-Fc-SB, ephrinB1-Fc-U, and ephrinB1-Fc-SP groups (fig. 4E inset).

Inhibition of Spinal MAPKs Prevented and Reversed Fos Protein Expression Induced by Intrathecal Injection of EphrinB1-Fc

The expression of Fos protein may also be a useful tool to examine the effectiveness of different analgesic regimens.[35–36] To further clarify the analgesic effect of inhibition of MAPKs on pain induced by intrathecal injection of ephrinB1-Fc, we investigated the effect of pretreatment or posttreatment with MAPKs inhibitors on spinal Fos protein expression induced by intrathecal injection of ephrinB1-Fc (0.5 μg). DMSO, SB (0.5 μg), U (0.5 μg), or SP (0.5 μg)

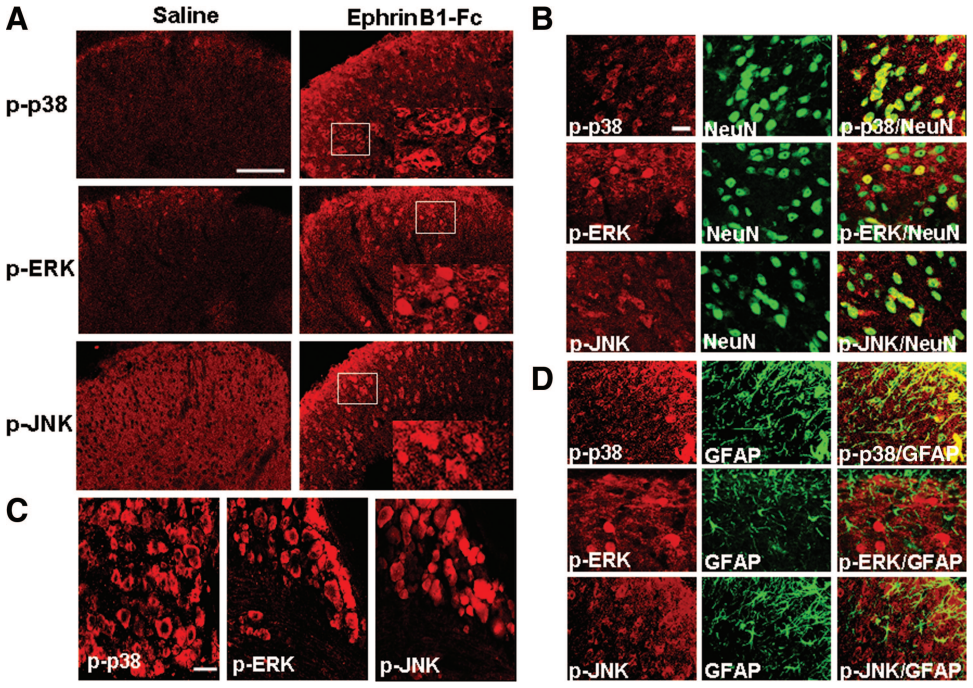

Fig. 3. Cellular localization of spinal phosphorylated mitogen-activated protein kinases. Double immunofluorescence staining of phosphorylated mitogen-activated protein kinases was performed with cell-specific markers: neuronal nuclei (NeuN) for neurons, glial fibrillary acidic protein (GFAP) for astrocytes at 30 min after intrathecal injection of ephrinB1-Fc (0.5 μg). Immunochemistry with p-p38, p-ERK, and p-JNK antibodies indicated that increased phosphorylated mitogen-activated protein kinases immunoreactivity levels were observed in the spinal cord dorsal horn (*A*) and dorsal root ganglion (*C*). (*B*) Double immunofluorescence staining showed that spinal p-ERK, p-p38, and p-JNK were expressed in neurons (colocalization with NeuN). (*D*) Double immunofluorescence staining showed that spinal p-p38 and p-JNK were expressed in astrocytes (colocalization with GFAP), and no colocalization between p-ERK and GFAP, indicating that p-ERK was not expressed by astrocytes. Scale bar = 100 μm.

was intrathecally administrated at 30 min before or after intrathecal injection of ephrinB1-Fc (0.5 μg), and spinal Fos protein expression was assayed at 2 h after ephrinB1-Fc injection. Both pre- and posttreatment with all three MAPKs inhibitors inhibited the spinal Fos expression induced by intrathecal injection of ephrinB1-Fc ($P < 0.01$; fig. 5).

Inhibition of Spinal MAPKs Decreased the Phosphorylation of the MAPKs Induced by Intrathecal Injection of EphrinB1-Fc

Our behavioral and molecular findings revealed that intrathecal injection of ephrinB1-Fc activated all three MAPKs pathways; however, inhibition of any one of the three MAPKs pathways could have almost completely prevented or reversed pain behaviors and spinal Fos expression induced by intrathecal injection of ephrinB1-Fc. To confirm whether inhibition of any one of the three MAPKs also decreased phosphorylation of the two other MAPKs as a consequence, DMSO, SB (0.5 μg), U (0.5 μg), or SP (0.5 μg) was intrathecally administrated at 30 min before (pretreatment) or after (posttreatment) intrathecal injection of ephrinB1-Fc (0.5 μg), and then, we assayed the phosphorylation of three MAPKs for each treatment in mice spinal cord at 1 h after pretreatment and 30 min after posttreatment. We found that intrathecal injection of any one of the three MAPKs inhibitors only decreased the phosphorylation of its target kinase but not of the other two MAPKs kinases in naïve mice (fig. 6A). However, pretreatment with p38 inhibitor decreased the phosphorylation not only of p38 but also of ERK and JNK by intrathecal ephrinB1-Fc. The similar results were also found in pretreatment with ERK and JNK inhibitors (fig. 6B). Furthermore, posttreatment with JNK inhibitor decreased the phosphorylation of three MAPKs induced by intrathecal injection of ephrinB1-Fc, and posttreatment with p38 inhibitor decreased the phosphorylation of p38 and ERK, not JNK, and posttreatment with ERK inhibitor only decreased the phosphorylation of ERK (fig. 6C). These results suggested that decreased activation of two other MAPKs by any one of the MAPKs inhibitors resulted from inhibition of interaction of intracellular signaling pathways, not from direct inhibition of other MAPKs by this MAPKs inhibitor.

EphBs Receptors Were Involved in Activation of Spinal MAPKs in the Neuropathic and Inflammation Pain Model

Spinal ephrinBs or EphBs signaling played a critical role in the development and maintenance of neuropathic and inflammation pain. To test whether spinal MAPKs activation contribute to the role of ephrinBs or EphBs signaling in neuropathic and inflammation pain, we analyzed the effect of intrathecal injection of EphB1-Fc, which can block ephrinBs or EphBs pathway, on expression of spinal p-MAPKs in CCI and formalin models (fig. 7). Consecutive intrathecal injection of EphB1-Fc on 1 h before CCI and days 1 and 2

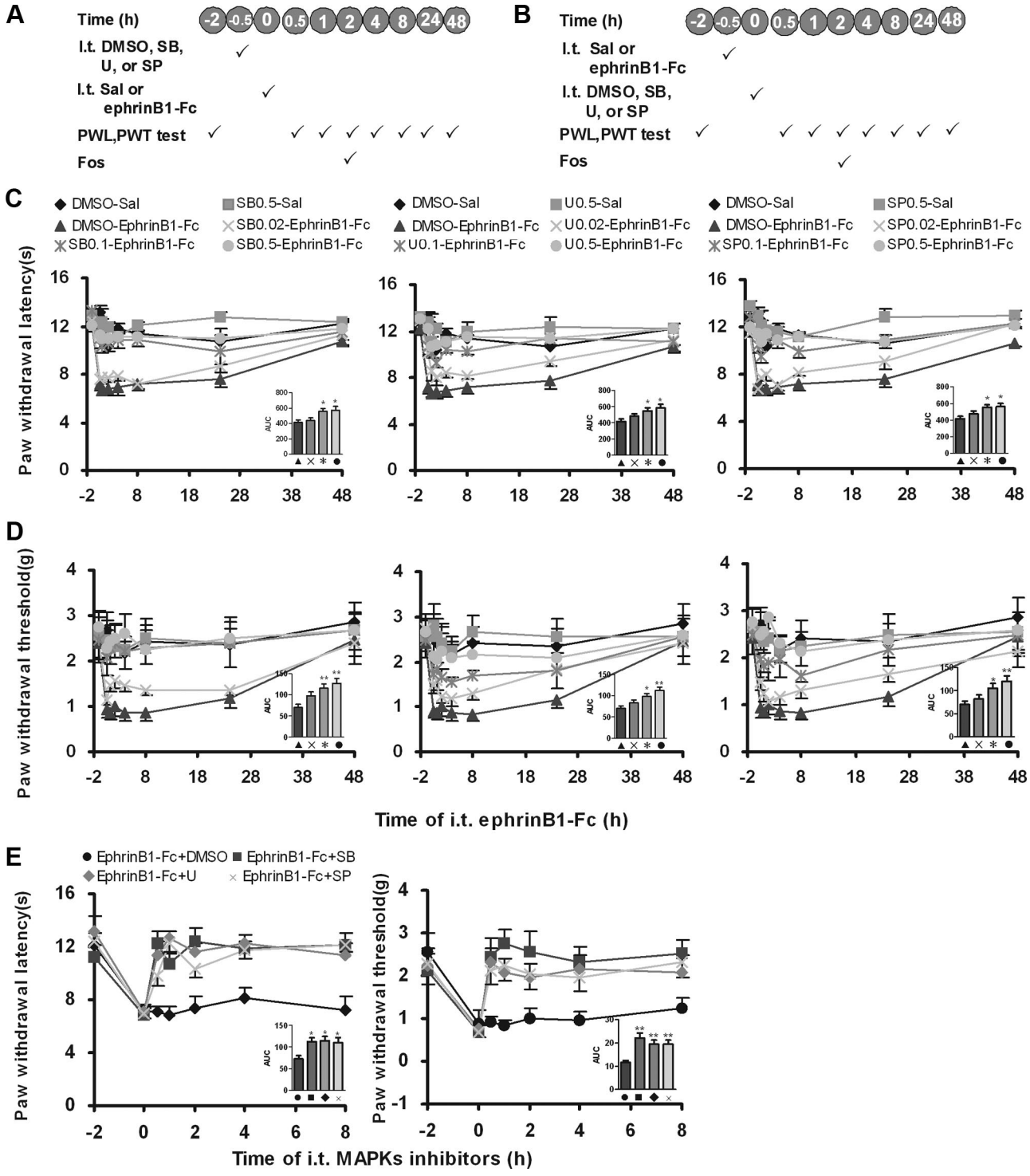

Fig. 4. Inhibition of spinal mitogen-activated protein kinases prevented and reversed pain behaviors induced by ephrinB1-Fc. (*A*) Schematic illustration of pretreatment experimental protocol. (*B*) Schematic illustration of posttreatment experimental protocol. (*C*, *D*) Intrathecal (i.t.) pretreatment with various dose (0.02, 0.1, and 0.5 μg) of p38 kinase inhibitor SB203580 (0.02, 0.1, and 0.5 μg) (SB0.02, SB0.1, and SB0.5), mitogen-activated protein/extracellular signal-regulated kinase inhibitor U0126 (0.02, 0.1, and 0.5 μg) (U0.02, U0.1, and U0.5), or JNK inhibitor SP600125 (0.02, 0.1, and 0.5 μg) (SP0.02, SP0.1, and SP0.5) prevented ephrinB1-Fc-induced thermal hyperalgesia (*C*) and mechanical alloynia (*D*) in a dose-dependent manner. Inset figures showed that, compared with dimethyl sulfoxide (DMSO)-ephrinB1-Fc group, the calculated area under the curve (AUC −2–48 h) in paw-withdrawal latency (PWL) and paw-withdrawal threshold (PWT) test was significantly increased in a dose-dependent manner in SB-ephrinB1-Fc, U-ephrinB1-Fc, and SP-ephrinB1-Fc group. * $P < 0.05$, ** $P < 0.01$ compared with DMSO-ephrinB1-Fc group. n = 8 mice in each group. (*E*) Posttreatment with SB (0.5 μg), U (0.5 μg), or SP (0.5 μg) reversed the established thermal hyperalgesia and mechanical allodynia by ephrinB1-Fc (0.5 μg). Inset figures showed that, compared with ephrinB1-Fc-DMSO group, the calculated AUC (−2–8 h) in PWL and PWT test was significantly increased in ephrinB1-Fc-SB, ephrinB1-Fc-U, and ephrinB1-Fc-SP group. * $P < 0.05$, ** $P < 0.01$ compared with ephrinB1-Fc-DMSO group. n = 8 mice in each group.

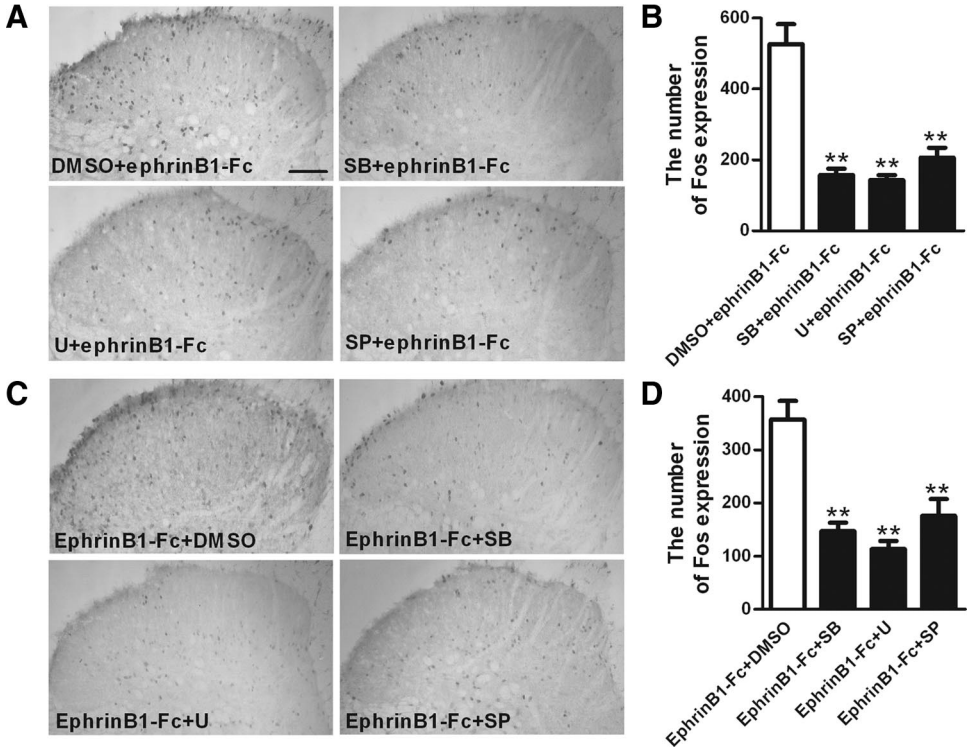

Fig. 5. Inhibition of spinal mitogen-activated protein kinases inhibited or reversed the increased expression of Fos protein by intrathecal injection of ephrinB1-Fc. Dimethyl sulfoxide (DMSO), 0.5 μg SB203580 (SB), 0.5 μg U0126 (U), or 0.5 μg SP600125 (SP) was intrathecally administrated at 30 min before intrathecal injection of ephrinB1-Fc (0.5 μg) in pretreatment experiment and at 30 min after intrathecal injection of ephrinB1-Fc (0.5 μg) in posttreatment experiment. Spinal Fos protein expression was assayed at 2 h after ephrinB1-Fc injection (see schematic illustration of experimental protocol in Fig. 4*A* and Fig. 4*B*). (*A*, *C*) The representative immunohistochemical staining, and (*B*, *D*) the quantitative data of Fos expression in the spinal cord of mice in pretreatment experiments or posttreatment experiments. ** $P < 0.01$ compared with DMSO + ephrinB1-Fc group or ephrinB1-Fc + DMSO group, n = 6 mice in each group, Scale bar = 100 μm.

after CCI (0.5 μg for each injection; see experimental protocol in fig. 7A) prevented the induction of thermal hyperalgesia and mechanical allodynia and this effect continued at least for 7 days after CCI ($P < 0.05$ or $P < 0.01$, EphB1-Fc + CCI *vs.* Sal + CCI; fig. 7C). On day 5 after CCI, a peak time point of pain behaviors, L_{4-5} spinal cord of some mice were extracted for Fos protein and p-MAPKs expression. Injection of EphB1-Fc significantly inhibited the increased expression of spinal Fos protein ($P < 0.01$ *vs.* Sal + CCI; fig. 7D) and p-MAPKs expression (Sal + CCI *vs.* Sal + Sham: $P < 0.001$ for p-p38 and p-ERK, $P < 0.05$ for p-JNK; EphB1-Fc + CCI *vs.* Sal + CCI: $P < 0.05$ for p-p38, p-ERK and p-JNK; fig. 7E) induced by CCI. Intrathecal injection of a single dose of EphB1-Fc (0.5 μg/5 μl) on fifth day after CCI (see experimental protocol in fig. 7B) reversed the established thermal hyperalgesia and mechanical allodynia, and this effect could last up to 8 h ($P < 0.05$, CCI + EphB1-Fc *vs.* CCI + Sal; fig. 7F). Spinal Fos protein ($P < 0.01$ *vs.* CCI + Sal; fig. 7G) and p-MAPKs (CCI + Sal *vs.* Sham + Sal: $P < 0.05$ for p-p38, p-ERK and p-JNK; CCI + EphB1-Fc *vs.* CCI + Sal: $P < 0.05$ for p-p38, p-ERK and p-JNK; fig. 7H) expression detected on 2 h after injection of EphB1-Fc also were markedly inhibited. We also got the similar antinociceptive effect when EphB1-Fc was injected in formalin model. Pretreatment with intrathecal injection of EphB1-Fc (0.5 μg/5 μl) at 30 min before formalin injection (see experimental protocol in fig. 8A) significantly reduced licking hind paw time in phase I and II responses (EphB1-Fc + Formalin *vs.* Sal + formalin, $P < 0.01$ at phase II and $P < 0.05$ at phase I; fig. 8B). The spinal Fos protein ($P < 0.01$ *vs.* Sal + formalin; fig. 8C) and p-MAPKs (Sal + formalin *vs.* Sal + Sal: $P < 0.05$ for p-p38, p-ERK, and p-JNK; EphB1-Fc + formalin *vs.* Sal + formalin: $P < 0.05$ for p-p38, p-ERK, and p-JNK; fig. 8D) expression, detected at 2 h and 30 min after injection of formalin, respectively, also was markedly inhibited.

Spinal NMDA Receptor Contributed to Activation of MAPKs by EphrinB1-Fc

NMDA receptor contributes to regulation of MAPKs activity in the spinal cord and many brain areas.[43,44] Activation of EphBs receptors could recruit and activate Src-family kinases, which then phosphorylate subunits of NMDA receptor and enhance activity of NMDA receptor.[45,46] Intrathecal administration of ephrinB2-Fc in adult rats, which can induce behavioral thermal hyperalgesia, led to NR2B tyrosine phosphorylation *via* Src family kinases.[14] Moreover, a great deal of evidence has confirmed that NMDA receptor was involved in central sensitization induced by nociceptive stimuli.[47–49] Thus, we speculated that NMDA receptor may be

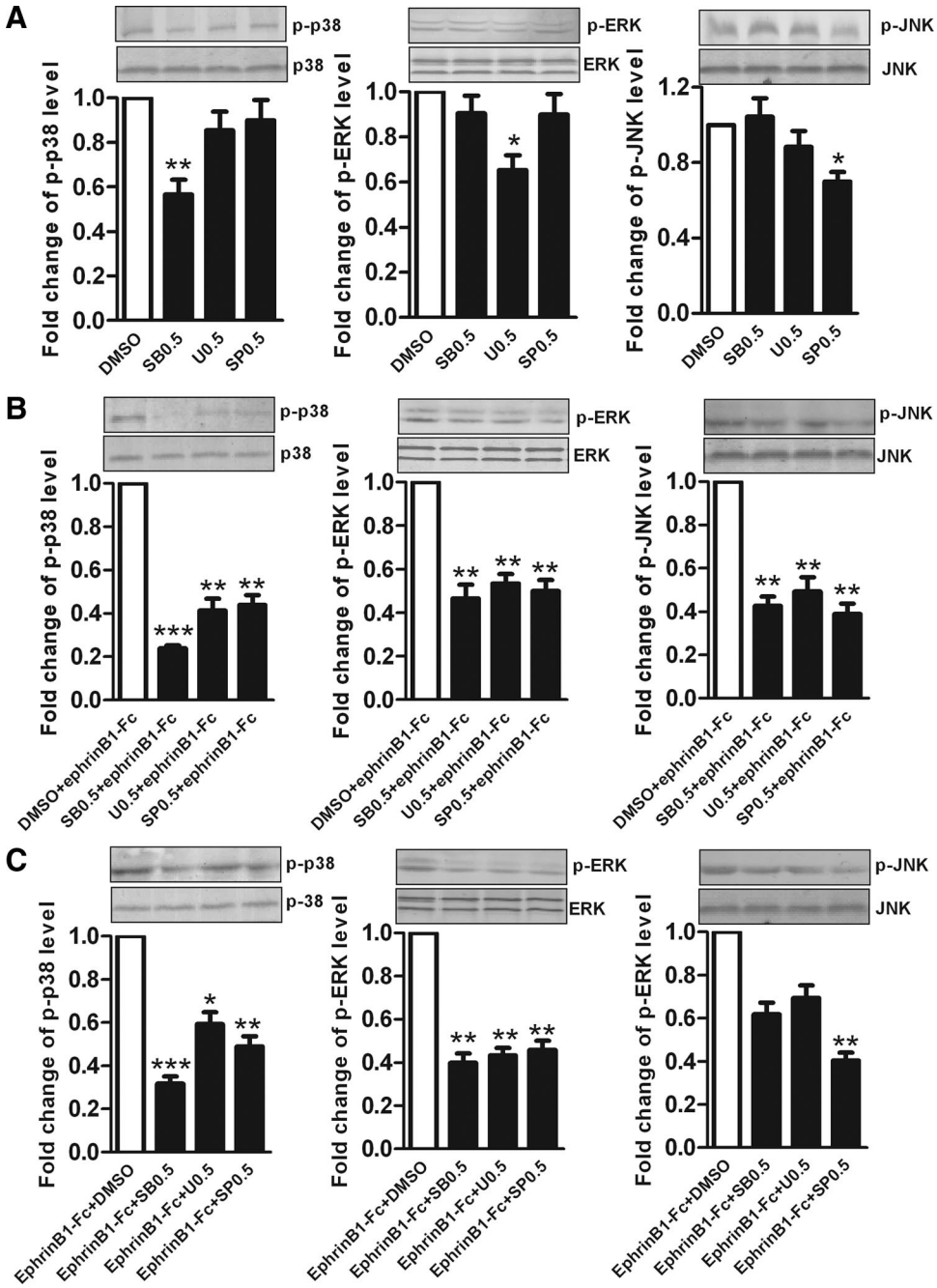

Fig. 6. Inhibition of spinal mitogen-activated protein kinases (MAPKs) decreased phosphorylation of MAPKs induced by intrathecal injection of ephrinB1-Fc in mice. Dimethyl sulfoxide (DMSO), 0.5 μg SB203580 (SB0.5), 0.5 μg U0126 (U0.5), or 0.5 μg SP600125 (SP0.5) was intrathecally administrated at 30 min before (pretreatment) or after (posttreatment) intrathecal injection of ephrinB1-Fc (0.5 μg). (*A*) The representative bands and the quantitative data for the expression of spinal p-p38, p-ERK, and p-JNK at 30 min after treatment with three different MAPKs inhibitors in naïve mice. * $P < 0.05$, ** $P < 0.01$, compared with DMSO group. (*B*) The representative bands and the quantitative data for the expression of spinal p-p38, p-ERK, and p-JNK at 1 h after pretreatment with three different MAPKs inhibitors. (*C*) The representative bands and the quantitative data for the expression of spinal p-p38, p-ERK, and p-JNK at 30 min after posttreatment with three different MAPKs inhibitors. The fold change for the density of each phosphorylated MAPKs normalized to total MAPKs for each sample. The fold change of phosphorylated MAPKs levels in DMSO + ephrinB1-Fc or ephrinB1-Fc + DMSO group was set at 1 for quantifications. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with DMSO + ephrinB1-Fc or ephrinB1-Fc + DMSO group, n = 5 mice in each group.

involved in activation of MAPKs by intrathecal injection of ephrinB1-Fc. To test this hypothesis, MK-801 (2.5 μg), an NMDA receptor antagonist, was injected 30 min before administration of ephrinB1-Fc. The L_{4-5} of spinal cord was collected to detect the expression of Fos protein and p-MAPKs at 2 h and 30 min, respectively, after injection of ephrinB1-Fc (see experimental protocol in fig. 9A). Our data indicated that pretreatment with MK-801 significantly prevented thermal hyperalgesia and mechanical allodynia (MK-801 + ephrinB1-Fc *vs.* ephrinB1-Fc, $P < 0.05$ from 0.5 to

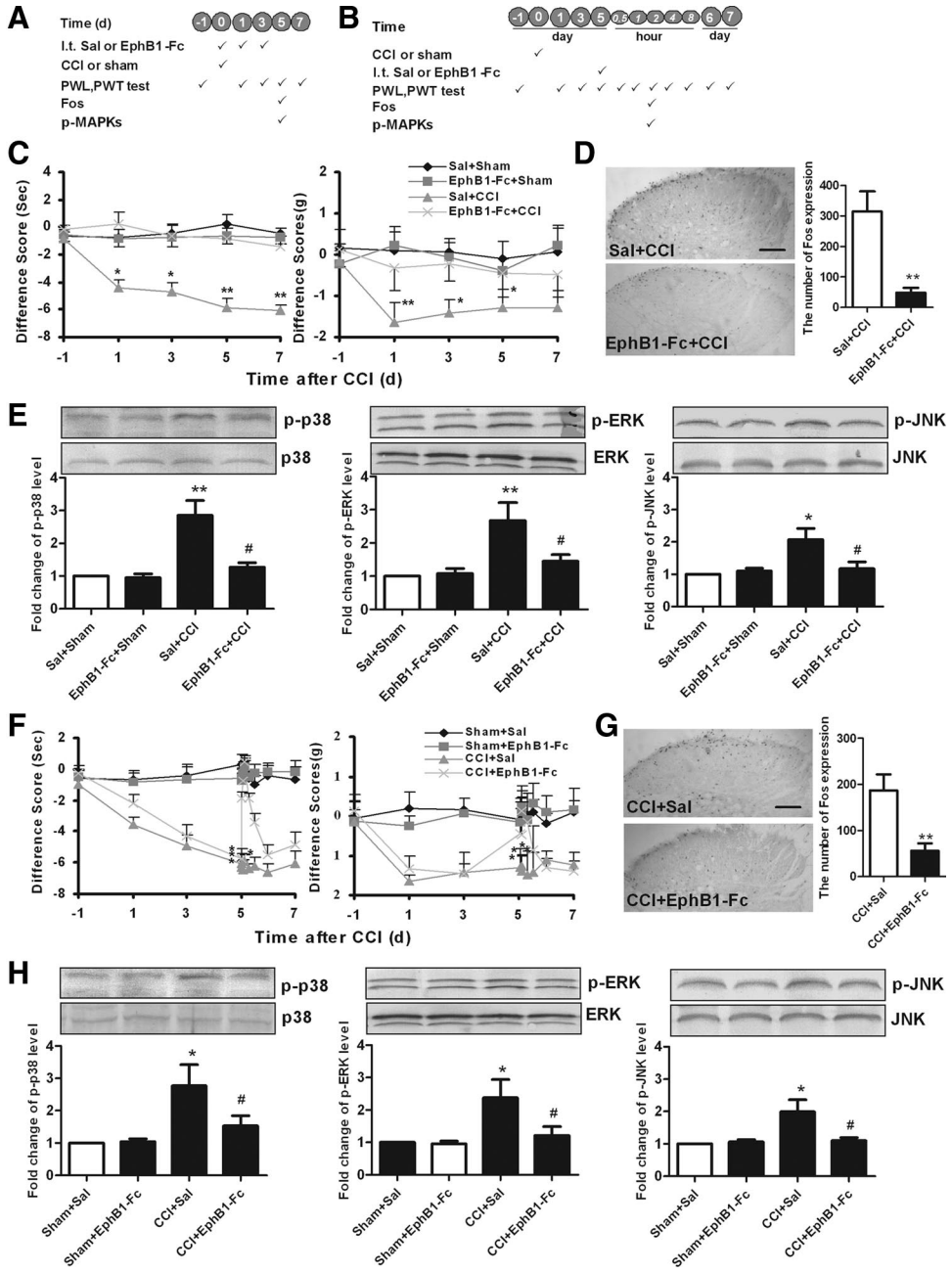

Fig. 7. Intrathecal (i.t.) pre- or posttreatment with EphB1-Fc inhibited chronic constrictive injury (CCI)-induced neuropathic pain and spinal Fos protein expression, accompanied by the decreased expression of spinal phosphorylated mitogen-activated protein kinases (p-MAPKs). (*A*) Schematic illustration of pretreatment experimental protocol. (*B*) Schematic illustration of posttreatment experimental protocol. (*C*) Pretreatment of EphB1-Fc (0.5 μg) prevented CCI-induced thermal hyperalgesia in paw-withdrawal latency (PWL) test and mechanical allodynia in paw-withdrawal threshold (PWT) test. * $P < 0.05$, ** $P < 0.01$, compared with EphB1-Fc + CCI, n = 10 mice in each group. (*D*) The representative immunohistochemical staining and the quantitative data for the decreased CCI-induced spinal Fos expression by pretreatment with EphB1-Fc. ** $P < 0.01$ compared with saline (Sal) + CCI group, n = 6 mice in each group, Scale bar = 100-μm. (*E*) The representative bands and the quantitative data for the decreased expression of spinal p-p38, p-ERK, and p-JNK by CCI after pretreatment with EphB1-Fc. The fold change for the density of each p-MAPKs normalized to total MAPKs for each sample. The fold change of p-MAPKs levels in Sal + Sham group were set at 1 for quantifications. ** $P < 0.01$ or * $P < 0.05$ compared with Sal + Sham group, # $P < 0.05$ compared with Sal + CCI group, n = 4 mice in each group. (*F*) Posttreatment of EphB1-Fc reversed CCI-induced thermal hyperalgesia and mechanical allodynia. * $P < 0.05$, compared with CCI + EphB1-Fc, n = 10 mice in each group. (*G*) The representative immunohistochemical staining and the quantitative data for the reversed CCI-induced spinal Fos expression by posttreatment with EphB1-Fc. ** $P < 0.01$ compared with CCI + Sal group, n = 6 mice in each group, Scale bar = 100-μm. (*H*) The representative bands and the quantitative data for the decreased expression of spinal p-p38, p-ERK, and p-JNK by CCI after posttreatment with EphB1-Fc. The fold change for the density of each p-MAPKs normalized to total MAPKs for each sample. The fold change of p-MAPKs levels in Sal + Sham group were set at 1 for quantifications. * $P < 0.05$ compared with Sham + Sal group, # $P < 0.05$ compared with CCI + Sal group, n = 4 mice in each group.

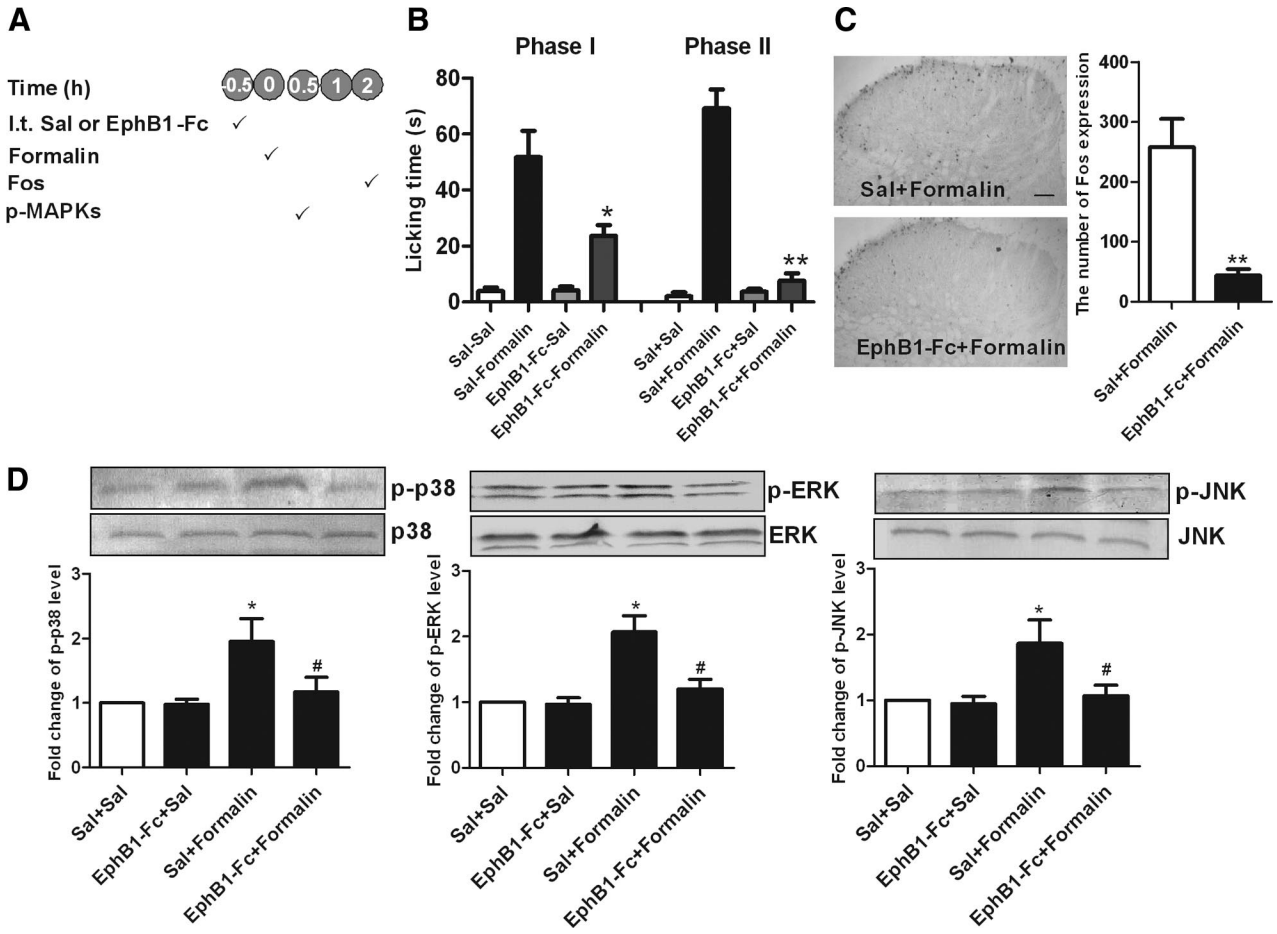

Fig. 8. Intrathecal (i.t.) pretreatment with EphB1-Fc inhibited formalin-induced inflammation pain and spinal Fos protein expression, accompanied by the decreased expression of spinal phosphorylated mitogen-activated protein kinases (p-MAPKs). (*A*) Schematic illustration of experimental protocol. (*B*) Pretreatment of EphBs receptors blocker EphB1-Fc (0.5 μg) decreased the time of licking/biting paw induced by formalin. * $P < 0.05$ or ** $P < 0.01$ compared with saline (Sal)-Formalin group, n = 8 mice in each group. (*C*) The representative immunohistochemical staining and the quantitative data for the decreased formalin-induced Fos expression by pretreatment with EphB1-Fc (0.5 μg), not saline, in the spinal cord of mice. ** $P < 0.01$ compared with Sal + formalin group, n = 6 mice in each group, scale bar = 100 μm. (*D*) The representative bands and the quantitative data for the decreased expression of spinal p-p38, p-ERK, and p-JNK by injection of formalin after pretreatment with EphB1-Fc (0.5 μg). The fold change for the density of each p-MAPKs normalized to total MAPKs for each sample. The fold change of p-MAPKs levels in Sal + Sal group was set at 1 for quantifications. * $P < 0.05$ compared with Sal + Sal group, # $P < 0.05$ compared with Sal + formalin group n = 4 mice in each group.

4 h; fig. 9B) and spinal Fos protein expression ($P < 0.01$ *vs.* ephrinB1-Fc; fig. 9C) and activation of spinal MAPKs ($P < 0.05$ *vs.* ephrinB1-Fc; fig. 9D) induced by ephrinB1-Fc.

Discussion

This study showed the following findings (1) spinal administration of ephrinB1-Fc produced a dose-dependent thermal hyperalgesia and mechanical allodynia, which was accompanied with the increased expression of spinal Fos protein and p-MAPKs; activated p38 and JNK were localized in both spinal neurons and astrocytes and p-ERK only in spinal neurons; (2) inhibition of spinal MAPKs prevented or reversed ephrinB1-Fc-induced hyperalgesia and spinal Fos protein expression; (3) blocking EphBs receptors alleviated formalin- and CCI-induced pain behaviors and inhibited activation of spinal MAPKs; and (4) spinal NMDA receptor mediated activation of spinal MAPKs by intrathecal injection of ephrinB1-Fc. These findings demonstrated a novel mechanism for ephrinBs/EphBs system involved in spinal nociceptive information modulation.

EphrinBs/EphBs, Neuronal Plasticity, and Pain

Central sensitization, an activity-dependent functional plasticity in spinal cord neurons, is one of the main causes of behavior hyperalgesia under pathologic conditions and has been under intensive investigation.[50–52] Activation of postsynaptic membrane receptors or ion channels, intracellular kinase cascades, and intranuclear gene expression contributes to the induction, development, and maintenance of central sensitization. Several studies have suggested that central sensitization related to pain and hippocampal long-term potentiation (LTP) associated with learning and memory may share certain mechanisms.[52,53] For example, activation of NMDA receptor and the subsequent associated intracel-

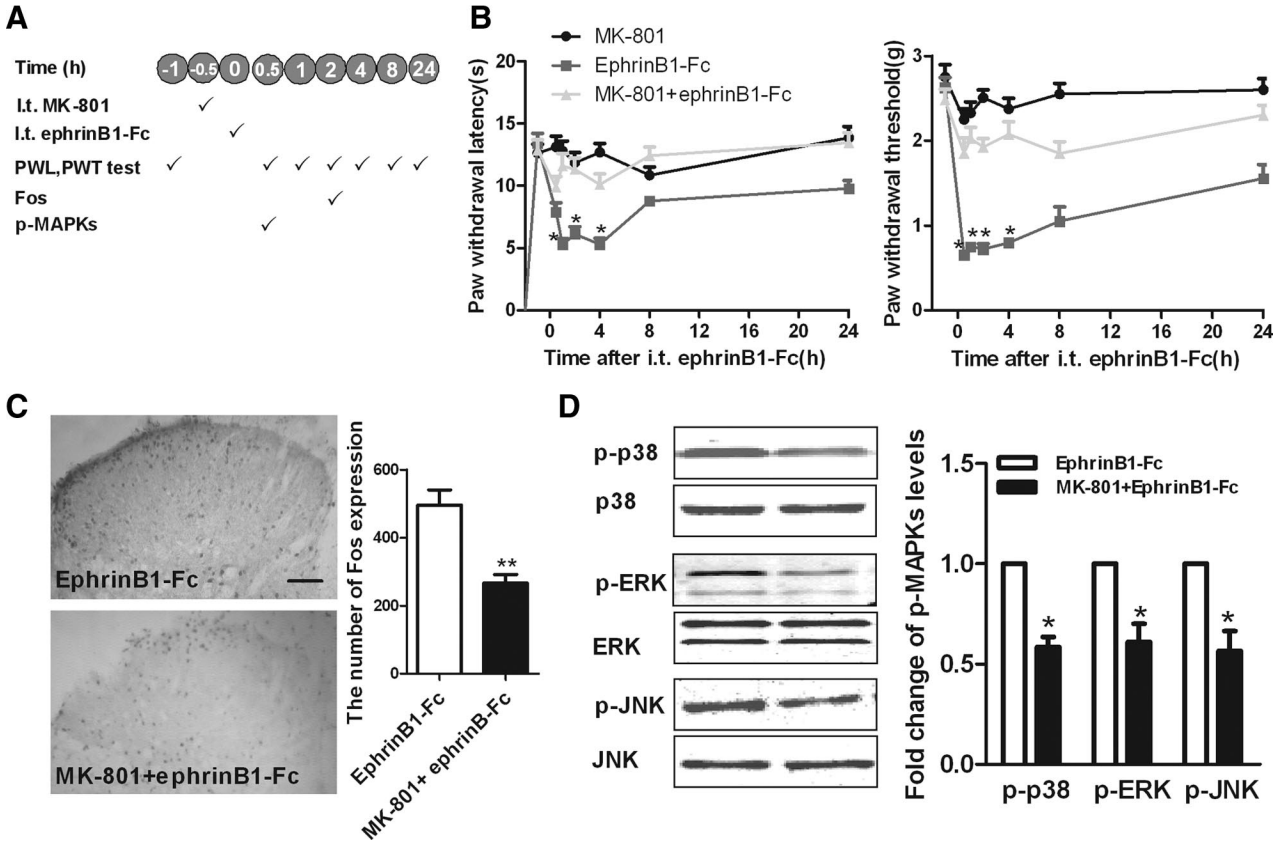

Fig. 9. Spinal *N*-methyl-D-aspartate (NMDA) receptor-mediated ephrinB1-Fc-induced hyperalgesia and increase of expression of phosphorylated mitogen-activated protein kinases (p-MAPKs). NMDA antagonist (+)-5-methyl-10,11-dihydro-SH-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801) (2.5-μg) was injected at 30 min before intrathecal (i.t.) injection of ephrinB1-Fc (0.5 μg). (*A*) Schematic illustration of experimental protocol. (*B*) The thermal hyperalgesia tested by paw-withdrawal latency (PWL) and mechanical allodynia tested by paw-withdrawal threshold (PWT) induced by ephrinB1-Fc (0.5 μg) were inhibited by pretreatment with MK-801 (2.5 μg). * $P < 0.05$ from 0.5 to 4 h, MK-801+ephrinB1-Fc *vs.* ephrinB1-Fc, n = 8 mice in each group. (*C*) The representative immunohistochemical staining and the quantitative data for the decreased ephrinB1-Fc-induced Fos expression by pretreatment with MK-801 (2.5 μg) in the spinal cord of mice. ** $P < 0.05$ compared with ephrinB1-Fc group, n = 6 mice in each group, Scale bar = 100 μm. (*D*) The representative bands and the quantitative data for the decreased expression of spinal p-p38, p-ERK, and p-JNK by intrathecal injection of ephrinB1-Fc (0.5 μg) after pretreatment with MK-801. The fold change for the density of each p-MAPKs normalized to total MAPKs for each sample. The fold change of p-MAPKs levels in ephrinB1-Fc group was set at 1 for quantifications. * $P < 0.05$, compared with ephrinB1-Fc group, n = 4 mice in each group.

lular signal transduction cascades are involved in the induction, development, and maintenance of synaptic plasticity in the spinal cord and hippocampus.

Both *in vitro* and *in vivo* studies had provided evidence that ephrinBs/EphBs system participated in synaptic plasticity and learning and memory in the adult hippocampus. EphB2 knockout mice show deficits in NMDA-dependent synaptic plasticity in the hippocampus, such as LTP and long-term depression.[46,54] Furthermore, mossy fiber LTP is also impaired by extracellular application of ephrinB1-Fc to inhibit EphB interactions with B-ephrins that are presumably localized presynaptically.[55] Now that there are some common cellular and molecular mechanisms between LTP in the hippocampus and central sensitization of nociceptive neurons in the dorsal horn, whether both share the ephrinBs/EphBs system mechanism is unknown. Our previous and current studies addressed this question. Application of EphB1-Fc, which can block EphBs receptors, onto the spinal cord significantly reduced the enhanced responses of wide dynamic range neurons in the dorsal horn to the innocuous and noxious stimulation in CCI rats by means of extracellular recordings in rat dorsal horn *in vivo*.[17] C-fibers-evoked LTP of the dorsal horn neurons may be associated with inflammation- and nerve injury-induced process of central sensitization and behavioral pain syndromes. We found that activation of ephrinBs-EphBs receptor signaling is required for induction of c-fibers-evoked LTP of dorsal horn neurons and lowers stimuli threshold of the LTP.[17]

Fos protein expression has been used as a maker for neuronal activation in the central nervous system and has been widely used as a tool in functional mapping of neuronal circuits in response to various defined stimuli. There is a positive correlation between the quantity of Fos protein expression and the degree of sensitization induced by nociceptive stimuli in spinal cord neurons. Long-term facilitation of c-fiber-evoked firing of wide dynamic range neurons in the spinal dorsal horn in response to conditioning stimulation of afferent fibers was accompanied with the frequency-depen-

dent increase of c-fos-labeled cells in superficial, intermediate, and deep laminae of the dorsal horn on the stimulated side.[56] Inhibition of tetanically sciatic stimulation-induced LTP of spinal neurons was also accompanied with the suppressed tetanic stimulation-induced spinal Fos expression.[57] In this study, we further confirmed the previous electrophysiologic findings by means of Fos protein expression. Spinal application of ephrinB1-Fc, which can activate EphBs receptors, induced a dose-dependent increase in spinal Fos protein expression. Blocking of EphBs receptors with intrathecal injection of EphB1-Fc also suppressed the increased expression of spinal Fos protein expression in CCI and formalin pain models. Although we cannot identify which EphBs subgroup was activated dominantly by ephrinB1-Fc, our study demonstrates clearly that activation of ephrinBs/EphBs system is required for development of the pain-related, long-lasting alterations of excitability and synaptic plasticity of spinal neurons, which lead to increased sensitivity to noxious stimuli (hyperalgesia) or nonnoxious stimuli (allodynia).

EphBs receptors transduce signals bidirectionally by interacting with membrane-anchored ephrinBs ligands expressed on adjacent cells. Application of ephrinB1-Fc reagent can activate EphBs receptors (forward signaling) and also prevent activation of endogenous ephrinBs ligands (reverse signaling). EphB1-Fc can disturb the signaling by the interaction of EphBs and ephrinBs and activate endogenous ephrinBs ligands. In this study, we could not confirm whether reverse signaling produced by ephrinBs activation was involved in hyperalgesia induced by activation of spinal ephrinBs/EphBs signaling. However, injection of EphB1-Fc could not induce any pain behavior and not affect basal pain threshold. It is possible that reverse signaling has no contribution to this process, or EphB1-Fc induces only weak agonist activity.

A great deal of evidence has indicated that spinal glial activation contributes to initiation and maintenance of central sensitization.[58] It has been widely demonstrated that expression of Eph receptors or their ephrin in astrocytes played multiple pivotal roles in various physiologic and pathologic processes such as hippocampus plasticity and spinal cord injury.[59–61] Ephrin/Eph signaling has been shown to be a potential regulator of neuron–astrocyte interactions mediating rapid structural and functional plasticity.[62,63] This study showed that intrathecal injection of ephrinB1-Fc induced activation of p38 and JNK in spinal astrocytes, indicating that ephrinBs/EphBs signaling in spinal astrocytes was involved in nociceptive information modulation in this pain model. However, further researches are required to address the mechanisms of spinal astrocytes involved in development and maintenance of pain behavior related to ephrinBs/EphBs signaling.

EphrinBs/EphBs, MAPKs, and Pain

Activation of intracellular kinase cascades and intranuclear gene expression plays a critical role in the development and maintenance of central sensitization. According to the structural features of EphBs receptors, MAPKs signaling may be a good candidate as its downstream effectors. As other receptor tyrosine kinases, EphBs receptors contain a highly conserved motif containing two tyrosine residues that are the major autophosphorylation sites. Activation of EphBs receptors induces autophosphorylation of specific tyrosine residues and creates docking sites for downstream molecules that contain Src homology 2 domain, such as Shc adaptor protein, and then Shc bridges EphBs to Grb2, which leads to activation of several downstream intracellular signaling, including the MAPKs cascade. On the other hand, activated EphBs receptors can also modulate the NMDA receptor function *via* a Src-dependent mechanism and then enhance NMDA receptor-mediated Ca^{2+}. This $[Ca^{2+}]i$ increase in turn directly facilitates the MAPKs pathways.

MAPKs, including p38, ERK, and JNK, are a family of serine/threonine protein kinases that transduce extracellular stimuli into intracellular posttranslational and transcriptional responses. It is well established that the MAPKs activation may be involved in the modulation of nociceptive information and peripheral and central sensitization produced by intense noxious stimuli through various routes.[18–23] Some studies report that ephrinBs/EphBs signaling regulates the activities of small GTPases of the Ras family and then activates the Ras/MAP kinase pathway in a wide variety of cell lines and tissues.[24–27,37,38] For example, ephrinB2 stimulation of EphB receptors in T cells also activates ERK1 and ERK2 MAP kinases. Ectopic expression of ephrinB1 in 293T cells leads to JNK activation.[37] EphB1 recruits c-Src and p52 Shc to activate MAPK/ERK and promotes chemotaxis.[64] These studies suggested that MAPKs signaling may mediate the role of ephrinBs/EphBs signaling in some physiologic and pathologic conditions. This study further supported and extended this view. We found that intrathecal injection of ephrinB1-Fc induced hyperalgesia accompanied by activation of spinal MAPKs. Blocking MAPKs prevents and reversed pain behavior and spinal Fos expression induced by intrathecal injection of ephrinB1-Fc. Furthermore, inhibiting spinal EphB1 receptor by intrathecal injection of ephB1-Fc reduces neuropathic and inflammation pain accompanied by the decreased expression of spinal MAPKs and Fos protein. These findings indicated that MAPK pathway was involved in spinal nociceptive information modulation related to ephrinBs/EphBs signaling. We further showed that pretreatment with NMDA receptor antagonist MK-801 prevented the activation of spinal MAPKs by ephrinB1-Fc accompanied by the reduced pain behavior. It suggested that NMDA receptor contributed to the activation of spinal MAPKs by ephrinB1-Fc. The activated MAPKs can translocate from cytoplasm into nucleus and, in turn, phosphorylate and increase the expression of transcriptional factors, such as cyclic adenosine 3′,5′-monophosphate response element binding and Fos and promotes the transcription of downstream genes. This point could reasonably interpret why ephrinB1-Fc-induced hyperalgesia

can last at least up to 24 h, when activation of MAPKs almost backed to baseline level at 8 h after intrathecal injection of ephrinB1-Fc.

This study found that intrathecal injection of ephrinB1-Fc activated all three MAPKs pathways; however, inhibition of any one of the three MAPKs pathways could have almost completely prevented or reversed pain behavior and Fos expression induced by intrathecal injection of ephrinB1-Fc. How to interpret this result? We believed that nociceptive stimuli activated a network of signaling molecules in the spinal cord. Each signaling molecule interacts with others in this network. Depending on the interaction of signaling molecules, this network keeps a high-level activation status, which could initiate and maintain pain behavior. Blocking any one of the interactions in this network will result in the decreased activation of whole network and pain behavior relief as a consequence. This viewpoint was supported by the results of the field of pain study. For example, lots of signaling pathways and molecules were involved in the development and maintenance of various types of pain. However, pain behaviors can almost completely be prevented or reversed by blocking any one of these signaling pathways or molecules. In agreement with this point, our study showed that inhibition of any one of the three MAPKs pathways also decreased the activation of the other two MAPKs.

Conclusion

This study demonstrates that activation of spinal ephrinBs/EphBs system induces hyperalgesia through an MAPKs-mediated mechanism. These findings may have important implications for exploring the roles and mechanisms of ephrinBs/EphBs system underlying central nervous system disease and for understanding the molecular basis for underlying physiologic and pathologic pain. In addition, it suggests that ephrinBs/EphBs pathway would be a new potential target for treatment of pathologic pain.

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