Activation of Src family kinases in spinal microglia contributes to formalin-induced persistent pain state through p38 pathway

Yong-Hui Tan[1*], Kai Li[1,2*], Xin-Yi Chen[1], Ye Cao[1], Alan R Light[3], Kai-Yuan Fu[1§]

[1] Center for TMD & Orofacial Pain, Peking University School & Hospital of Stomatology, Beijing 100081, P R China

[2] Department of General Dentistry II, Peking University School & Hospital of Stomatology, Beijing 100081, P R China

[3] Department of Anesthesiology, University of Utah, Salt Lake City, Utah 84132-2304, USA

* These authors contributed equally to this work

[§] Corresponding author: Kai-Yuan Fu, DDS, PhD

Center for TMD & Orofacial Pain

Peking University School & Hospital of Stomatology

Zhong Guan Cun South Avenue 22, Beijing 100081, P R China

Email: kqkyfu@bjmu.edu.cn; Tel: 86 10 62179977; Fax: 86 10 62173402

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Abstract

Protein tyrosine phosphorylation has been implicated in normal and pathological functions, such as cell proliferation, migration and differentiation. Recently, some studies have shown that Src family kinases (SFKs) were involved in neurological disorders and neuropathic pain states in which microglial activation plays a role. In the formalin test, we have reported that microglia undergo at least two distinct stages of activation on the basis of signaling events regarding p38 mitogen-activated protein kinases (MAPK). Here, we investigated the involvement of SFKs signaling in formalin pain animal model, and the association with p38 MAPK. Our results showed that SFKs were activated in the spinal microglia beginning 1 day after peripheral formalin injection, lasting for 7 days. Pretreatment with SFK specific inhibitor PP2 could not inhibit formalin induced spontaneous pain behaviors. However, PP2 inhibited formalin injury induced persistent mechanical hyperalgesia, and reversed microglial phospho-p38 expression as well using immunohistostaining and Western blot at day 3 and 7 after injection. Our results suggested that the activation of the Src/p38MAPK signaling cascade in spinal microglia contributed to late stage persistent mechanical hyperalgesia evoked by formalin injection into the paw.

Perspective: This study presents unique properties of spinal microglial activation in a pain animal model. This finding could potentially help clinicians to further understand the contributions of spinal microglia to acute and persistent pain state.

Key words: Chronic pain; Formalin; Microglia; Src family kinases; p38 mitogen-activated protein kinase

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Introduction

Src family kinases (SFKs), one group of non-receptor protein tyrosine kinases implicated in the regulation of cell proliferation, migration, differentiation and survival, are related to immune receptors and also demonstrated cross-talking with other signaling pathways.[24,31] In the central nervous system (CNS), SFKs play a crucial role in synaptic transmission and plasticity.[18] SFK activation is also involved in signaling cascades in sensory ganglia and spinal cord that regulate pain perception.[9,15] Recently, some studies demonstrated that SFKs mediate microglial functions related to many neurological disorders.[2,19,33]

Microglia are the resident immune cells mediating a number of pathological events in CNS including the involvement in playing central sensitization in persistent pain states. Mitogen-activated protein kinases (MAPKs) in microglia have been demonstrated to play important roles in these pathological events.[10,16,17] Several members of SFKs are expressed abundantly under different conditions of microglial activation, and multiple lines of evidence have indicated that SFKs are intermediates in various pathways leading to MAPK activation in pathology or normal physiological events.[1,20,21,34] Katsura et al.[19] found that nerve injury induced robust SFK phosphorylation in spinal microglia. Intrathecal injection of an SFK inhibitor reversed nerve injury induced hyperalgesia, as well as the activation of one MAPK pathway (ERK) in dorsal horn microglia. These data indicate that MAPK signaling in activated microglia may be mediated via SFK activation. Thus, SFKs represent key components making up the microglial activation cascade.

In the formalin pain test, we reported robust microglial activation with morphological changes in the spinal cord dorsal horn,[5] and recently found that microglia may undergo at

least two distinct stages of activation on the basis of their morphological and immunological changes.[22] At later times (second stage, 3 days to weeks) microglia acquire the morphological and cellular surface immune phenotype changes as well as a delayed second period of upregulation of p38 MAPK activation.

Here, we hypothesized that activation of SFKs in spinal microglia contributed to peripheral formalin injury-induced pain state through p38 pathway. This hypothesis is supported by the findings that SFKs were activated in the spinal microglia at a late stage following peripheral formalin injection. Furthermore, we show that peripheral formalin injection induced second stage of p38 activation in spinal microglia via SFK activation and that this activation of the Src/p38MAPK signaling cascade contributed to persistent mechanical hyperalgesia evoked by formalin injection into the paw.

Materials and Methods

Animals and treatments

Male adult Sprague-Dawley rats weighing 200 g to 225 g (Vital River Laboratory Animal Technology Co. Ltd, Beijing) were used, and all protocols for the experiments were approved by the Animal Care and Use Committee of Peking University Health Science Center and certified that the care and use of animals conformed to applicable national/international guidelines. All rats were housed at temperature of 23°C (± 1°C) on a 12-hour light/dark cycle with free access to food and water. Experimental rats in the formalin model group received subcutaneous injections of 100 μl 5% formalin (diluted in 0.9% saline) into the plantar surface of the right hind paw. The control group rats were injected with 100 μl 0.9% saline

instead of formalin or received no treatment. Survival times were 30 minutes, 60 minutes, 6 hours, 1 day, 3 days, 7 days, and 14 days post-injections, and lumbar spinal cord was taken for immunohistochemical and western blot analysis.

Chronic lumbar intrathecal (i.t.) catheters were implanted according to the procedure described by Størkson et al.[29] Briefly, under adequate anesthesia with sodium pentobarbital, a polyethylene catheter (20 cm; PE-10, Stoelting, Wood Dale, IL) was introduced in the subarachnoidal space via the L5/6 intervertebral space and advanced rostrally 3.0~3.5 cm in order to reach the lumbar enlargement. The catheter was fixed to the fascia, tunneled subcutaneously on the back of the rats, and its proximal end was externalized in the occipital region. Animals were allowed to recover for at least 5 days after implantation, and no rat was found to have signs of neural dysfunction after surgery in this experiment. The SFK inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; Calbiochem, La Jolla, CA) or the inactive analogue PP3 (4-amino-7-phenylpyrazol[3,4-d]pyrimidine; Calbiochem) was delivered intrathecally (with the same volume of 10 μl) and the catheter flushed with 12 μl of saline. Both of the agents were dissolved in dimethylsulfoxide (DMSO), and diluted in 0.9% saline when used.

Behavioral analysis

Acute spontaneous nociceptive behavior

The animals were placed in a 30×30×30-cm clear Plexiglas chamber with a mirror below the surface to allow an unobstructed view of the paws. To allow familiarization with surroundings, rats were habituated in the test chambers singly for 20 min for 3 days. On the test day, following 5% formalin injections, rats were returned to the observation box

immediately, and the behaviors were monitored using a video camera for 60 min. The number of flinches was recorded for 5-min periods, and added up during phase I (0~10 min) and phase II (10~60 min) respectively. PP2 (5 μg), PP3 (5 μg) or vehicle (50% DMSO, diluted by saline) was given 20 min prior to the formalin injection, 6 rats for each group.

Secondary persistent mechanical hyperalgesia

Animals received subcutaneous injections of 100 μl 5% formalin into the plantar surface of the right hind paw. PP2 (5 μg), PP3 (5 μg) or vehicle (50% DMSO) was first delivered intrathecally 20 min prior to the formalin injection (6 rats per group), and repeated once a day at 4 P.M. until day 7. The mechanical threshold for nociceptive response was conducted as described previously,[27] and all tests were conducted before treatment and on day 1, 3, 5, 7 pre drug delivery under blind conditions. Briefly, the rat was habituated to standing on its hind paws and against the tester's gloved hand. The withdrawal threshold of the hind paw in response to mechanical stimulation was determined by using a hand-held force transducer (electronic anesthesiometer, IITC Life Science, Woodland Hills, CA) adapted with 0.5-mm diameter polypropylene rigid tip. The area tested was the dorsal surface of right hind paw, between the third and fourth metatarsals. The investigator was trained to apply the tip perpendicular to the central area of the hind paw with a gradual increase in pressure. The force in grams needed to elicit clear paw withdrawal indicative of nociceptive response was recorded four times for each animal at 1-min intervals, and the average of the four values was used as the withdrawal threshold. The percentage change from the baseline threshold was also calculated for each one. All tests were performed by a single investigator who was blinded to treatment groups.

Immunohistochemistry

Animals (3 to 5 rats at each time point) were anesthetized with an overdose of pentobarbital sodium (100 mg/kg, i.p.) and euthanized by transcardiac perfusion with 250 ml body temperature 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 300 ml ice-cold 4% paraformaldehyde/4% sucrose in 0.1 M PB, pH 7.4. After perfusion, the lumbar spinal cord (L4~5) was removed, postfixed in 4% paraformaldehyde fixative for 4~6 hours and then placed in a 30% sucrose solution (in 0.1 M PB) over two-night at 4°C. Thirty micron thick spinal cord sections were cut transversely on a cryostat for free-floating immunohistochemical staining. The sections were blocked with 4% normal goat serum (NGS) and then incubated for 48 hours at 4°C in the phospho-Src-family or phospho-p38 antibody (1:200, Cell Signaling Technology, Beverly, MA). Binding sites were visualized with FITC-conjugated secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA).

For double immunofluorescence, tissues were incubated with a mixture of primary antibodies with monoclonal neuron-specific nuclear protein (NeuN, neuronal marker, 1:5000; Chemicon, Temecula, CA), glial fibrillary acid protein (GFAP, astrocyte marker, 1:200; NeoMarkers, Fremont, CA) and OX-42 (CD11b, microglia marker, 1:200, Serotec, Indianapolis, IN). Following the incubation, spinal sections were washed and incubated for 1.5 hours at room temperature in a mixture of FITC- and TRITC-conjugated secondary antibodies (Jackson ImmunoResearch). The stained sections were examined with a fluorescence microscope (Olympus BX51, Tokyo, Japan), and images were captured with a CCD spot camera. The image enhancement was performed by using Adobe Photoshop 12.0. In our experiments, replacement of primary antibody by normal serum or PBS resulted in no

staining.

Western blot analysis

Rats (4 rats at each time point) were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) then decapitated. The spinal cord segments L4~5 (lumbar enlargement) ipsilateral to the injection were removed rapidly and homogenized in RIPA buffer (Cell Signaling Technology; supplemented with 1mM PMSF, phosphatase and protease inhibitor cocktail, Sigma). The homogenate was centrifuged at 15,000 g for 40 min at 4°C. The protein concentration of tissue lysates was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). Aliquots (30 μg protein) were subjected to 12% SDS-PAGE, and transferred electrophoretically to PVDF filters (Millipore, Bedford, MA). After blocking with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 hour in room temperature, the membranes were incubated with antibody to phospho-Src-family or phospho-p38 and total Src or p38 (Cell Signaling Technology; all diluted 1:1000 in blocking buffer) overnight at 4°C. After washing, the antibody-protein complexes were probed with HRP-conjugated secondary antibody (1:10000, Jackson ImmunoResearch), developed in ECL solution for 3 min, and exposed onto Kodak hyperfilms. The intensity of immunoreactive bands was quantified using NIH ImageJ 1.38 software, normalized to the density of internal control β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) and expressed as fold changes as compared to control group.

Statistical analysis

All data are presented as mean ± SEM. Statistical significance was calculated using Student's t-tests or one-way ANOVA for western blot analysis or ANOVA for repeated

measures for behavior test using SPSS software (Version 16.0, SPSS Inc). Differences were considered to be significant when the critical value reached a level of $P < 0.05$.

Results

Intraplantar formalin injection induced a long-lasting increase in SFK phosphorylation in the spinal microglia

We used anti-p-Src family (Tyr416) antibody to detect phospho-activation of the signaling molecules. In the spinal dorsal horn of naïve and saline injected control rats, there was no or very few p-SFKs-immunoreactive (IR) cells (Figs 1A,B). However, the increase of p-SFKs-IR was detected in the L4-L5 dorsal horn on the ipsilateral side at day 1 and significantly observed at day 3 after formalin injection (Fig 1C); no change was found in the ventral horn. Many p-SFKs-IR cells were found in the superficial and medial portion of the L4-L5 spinal dorsal horn ipsilaterally, and a few positive cells also were noted on the contralateral side (Fig 1D). Double-labeling immunohistochemistry was performed to identify types of the p-SFKs-IR cells. The majority of the p-SFKs colocalized with OX-42 (microglia) but not NeuN (neuron) or GFAP (astrocyte) (Figs 1E-G). SFK activation was confirmed by Western blot (Fig 2). The increase of the phosphorylated protein was significant at day 1, continued for over 7 days, and became weaker 14 days after formalin injection. Total SFK protein level was not changed statistically (data not shown). Interestingly, no significant change was observed in p-SFKs as determined by immunohistochemistry or Western blot at the early time (within several hours) following formalin injection.

Effects of SFK inhibitor PP2 on formalin-evoked early biphasic nociceptive behavior

To test involvement of SFK activation in the formalin-evoked nociceptive behaviors, we suppressed SFK activation in the spinal cord by applying Src family protein tyrosine kinase inhibitor PP2 via a lumbar subarachnoid catheter, and counted hind paw flinching numbers within one hour following formalin injection. PP3 (a truncated, non-SFK affecting form of PP2) served as negative control. Both of the reagents had no effect on basal mechanical sensitivity (data not shown). PP2 (5 μg) or PP3 (5 μg) was delivered 20 minutes before the formalin injection. As Figure 3 indicates, PP2 did not influence the flinching activity. PP3 results were similar to that of the vehicle control (Fig 3).

SFK activation and formalin-evoked persistent mechanical hyperalgesia

We reported previously that the formalin test could produce a third phase of nociceptive behavior consisting of mechanical hyperalgesia beginning days after formalin injection and lasting for several weeks.[6,22] To test involvement of SFK activation in the late formalin-evoked persistent mechanical hyperalgesia, PP2 (5 μg) or PP3 (5 μg) was delivered once a day for 7 successive days, with the first administration 20 minutes before the formalin injection. We found that intrathecal PP2, but not PP3, effectively reversed formalin-induced long-term mechanical hyperalgesia at days 3, 5 and 7 as compared with vehicle-treated control (Fig 4A). Inhibition of SFK activation by PP2 was confirmed by Western blot (Fig 4B).

Inhibition of SFK activation reversed the second long-lasting p38 MAPK activation induced by intraplantar formalin injection

We previously reported that peripheral formalin injection induces 2 stages of microglial activation: an early stage associated with inflammation or immediate injury discharge, and a

late stage associated with nerve injury.[22] Since inhibition of SFK activation suppressed the formalin-induced late stage of persistent mechanical hyperalgesia, the phospho-p38 (p38 activation state) expression was assessed in the presence of SFK inhibitor PP2. As expected, PP2 reversed the formalin-induced p38 activation as observed with immunohistostaining and Western blot at day 3 and 7 after injection. As controls, we also demonstrated that the expression of p-p38 was significantly increased in the vehicle and PP3 (negative control) groups (Fig 5).

Discussion

The intracellular signaling cascades involving SFKs play critical roles in various cellular responses including metabolism, proliferation and migration. Here, we show that SFKs in the spinal microglia are activated days after peripheral formalin injection and contribute to the third phase (late stage) of nociceptive behaviors in formalin pain model. Our results further suggest that SFK activation is involved only in the late stage of long-lasting pain state. Moreover, peripheral formalin injection induces p38 activation in spinal microglia via SFK activation and this activation of the Src/p38MAPK signaling cascade contributes to the late long-term mechanical hyperalgesia evoked by formalin paw injection.

Microglia are highly dynamic cells that undergo rapid cellular remodeling that changes throughout the progression of long-lasting pain

Spinal cord microglia can be activated by peripheral inflammation and nerve injury. Activated microglia change in morphology from ramified to amoeboid, and concomitantly increase the expression of microglial markers such as CD11b and MHC antigens.[3,4,7] These

dynamic changes usually take days.[3,5,8,11,13,23,25] We have previously reported that spinal microglia activation can occur in minutes after peripheral formalin injection as indicated by the phosphorylation of p38 MAPK. Later, activated microglia changed their morphology and up-regulated several immune markers (CD11b, CD45, and MHC class I) beginning from 1 to 3 days after injection.[5,7] Thus microglia may undergo at least 2 distinct stages of activation, one very early and the other days later in the formalin pain animal model. Intriguingly, we show here that SFKs are activated only beginning 1 day after peripheral formalin injection. Moreover, SFK inhibitor PP2 could reverse the third phase of prolonged hyperalgesia, but not the acute Phase I and Phase II nociceptive behaviors that occur minutes after the formalin injection. These data indicate that each phase of formalin test may rely on distinct cellular and molecular mechanisms for microglia at spinal cord level.

It has been demonstrated that Src is expressed in spinal dorsal horn neurons and contributes to CFA-induced inflammatory pain.[9] However, in peripheral nerve injury model using L5 spinal nerve ligation, the cells expressing p-SFKs are microglia rather than neurons or astrocytes in the dorsal horn.[19] Tsuda et al.[32] also observed that SFKs were activated in spinal microglia in the neuropathic pain model created by permanently damaging afferent nerve fibers, but not in the inflammatory pain model caused by CFA that does not cause extensive damage to afferent nerves. As we have previously reported, the formalin test produces both a short-term inflammatory pain response, and longer-term nerve injury pain responses.[6,35] Thus, in the present study, SFKs may be activated in microglia of spinal cord specifically by the nerve injury, rather than by the inflammatory event. This may indicate that different specific molecules participate in microglial activation in distinct pathologic

conditions.

Src/p38MAPK activation in microglia contribute to formalin-induced persistent hyperalgesia

Microglia are thought to be the main cell type dedicated to innate immunity in the CNS. Their functions in immune surveillance depend on intracellular signaling cascades. The bulk of these pathways are initiated by protein tyrosine kinases, the most important of which is SFKs. SFKs have been implicated as upstream molecules activated early after surface receptor activation leading to activation or inhibition of the immune response.[24] SFKs are also considered important activators of other kinase families in TLR (Toll-like receptor) family, playing important roles in regulating cytokine expression.[26] Innate immune signaling occurs in resident microglia under the condition of inflammation, degenerative disease and pathological pain in CNS. CD45, MHC class I and II can be activated in spinal microglia by some pain animal models.[7,12,14,30] Intrathecal delivery of the immunosuppressive agents can inhibit the cellular immune response of activated microglia and prevent the development of pain after nerve injury.[28] SFKs expression in activated microglia may indicate that immune responses in the spinal cord were initiated.

In the present study, we found that a Src family inhibitor PP2 reversed formalin-evoked long-lasting mechanical hyperalgesia as well as p38 MAPK activation in spinal microglia, suggesting that Src/p38MAPK signaling mediates long-lasting hyperalgesia. Thus, we hypothesize that the chain of events leading to long-lasting hyperalgesia includes very early microglial activation indicative of p38 MAPK phosphorylation evoked by afferent nociceptors followed days later by the activation of SFKs. This late activation of SFKs may

be caused by signals from nerves killed or injured by the formalin injection. The SFKs may then cause a second wave of phorphorylation of p38 MAPK that leads to the second, long-lasting hyperalgesia typical of tissue damage that involves extensive nerve injury.

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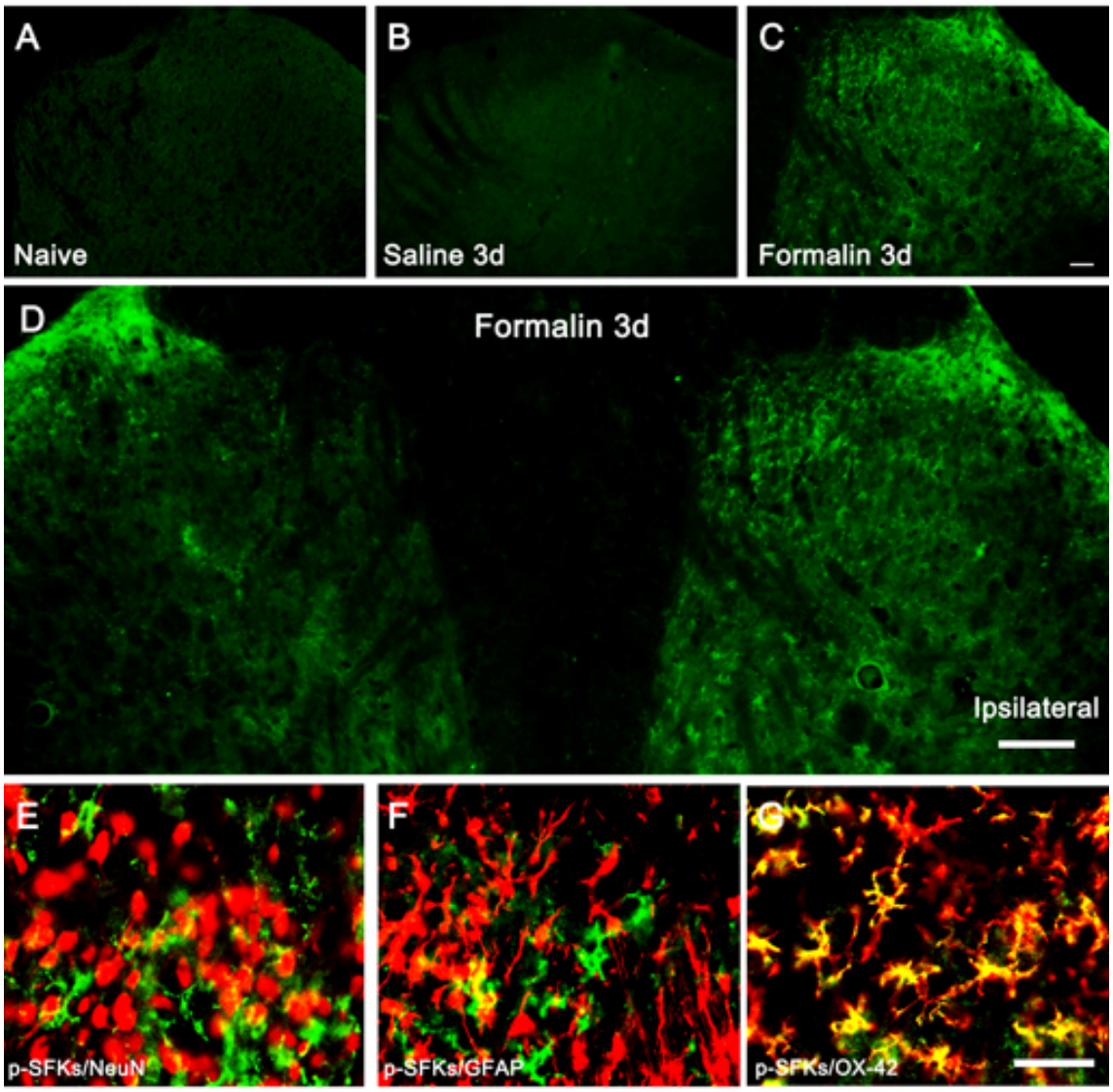

Figure 1 Peripheral formalin injection induced SFK activation in the lumbar spinal dorsal horn. **A-C**, Representative immunostaining images of p-SFKs expression in the spinal dorsal horn from naïve, saline and formalin-injected group at day 3 after injection. Scale bar: 50 μm. **D**, p-SFK immunoreactivity increased in the ipsilateral side of the dorsal horn at day 3 after formalin injection. Scale bar: 100 μm. **E-G**, Merged images of double immunofluorescent labels of p-SFKs (green) with markers of neurons (red, NeuN), astrocytes (red, GFAP), and microglia (red, OX-42), p-p38 was expressed in most microglia but rarely in neurons or astrocytes. Scale bars: 50 μm.

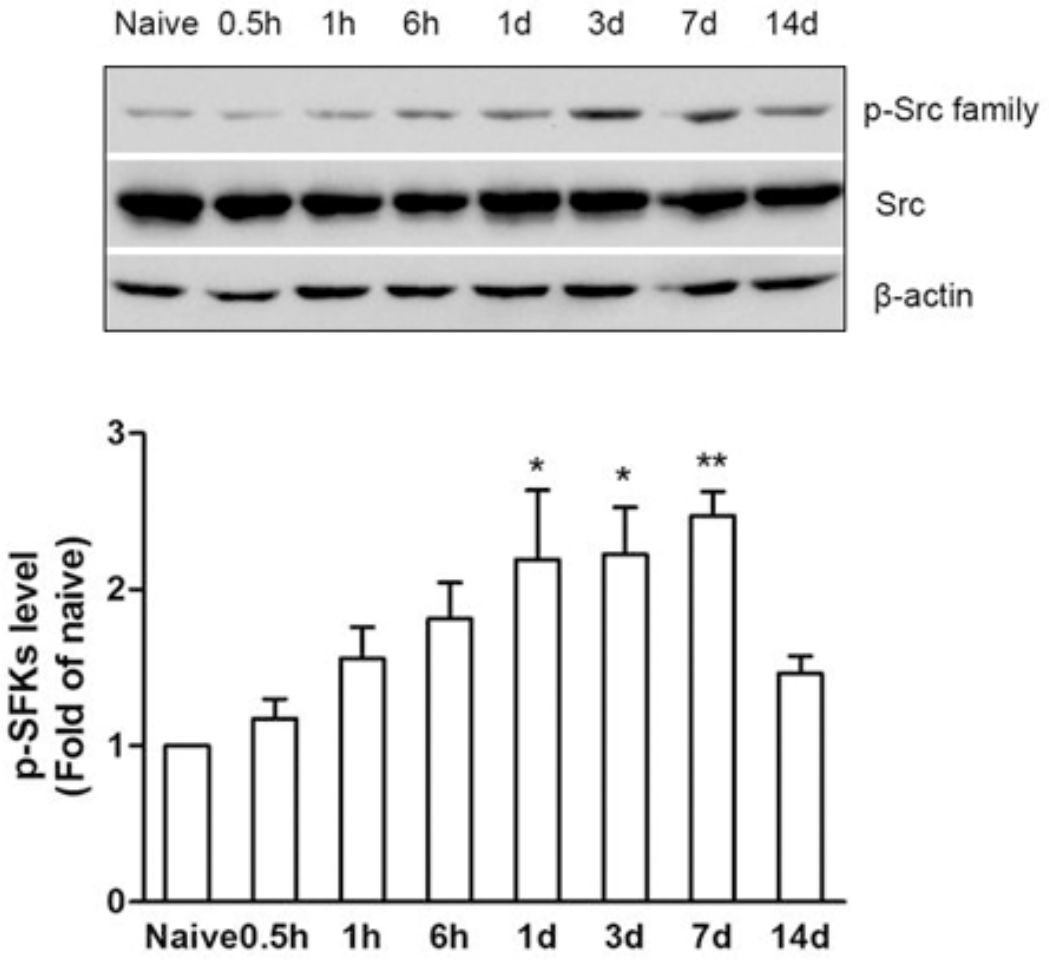

Figure 2 Representative bands and quantification of Western blot analysis showed persistent increased p-SFKs protein level in the lumbar spinal cord after formalin injection. Quantification of p-SFKs level were normalized against a control protein, β-actin. **P* < 0.05, ***P* < 0.01, one-way ANOVA and Tukey post-hoc test, compared with the naïve (uninjected) control, n = 4.

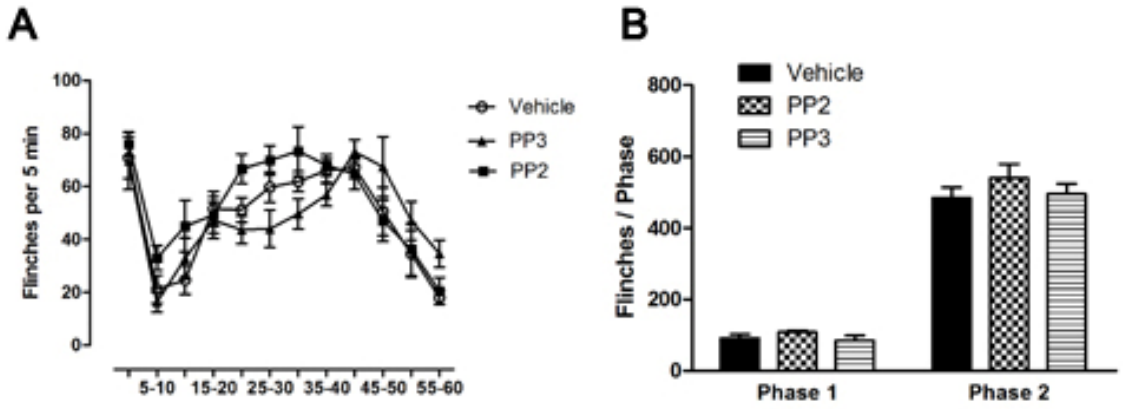

Figure 3 Effects of the SFK inhibitor PP2 on formalin-induced the early biphasic flinching behavior. **A**, Time courses of flinching behavior number from three different treatment groups following peripheral 5% formalin injection. Each point was the mean number of flinches (± SEM) (n = 6) per 5 min epoch. **B**, The bars showed total numbers of flinches during the two phases in rats treated with vehicle, PP2 and PP3. No significance compared to vehicle group, n = 6, one-way ANOVA, followed by a Tukey post-hoc test.

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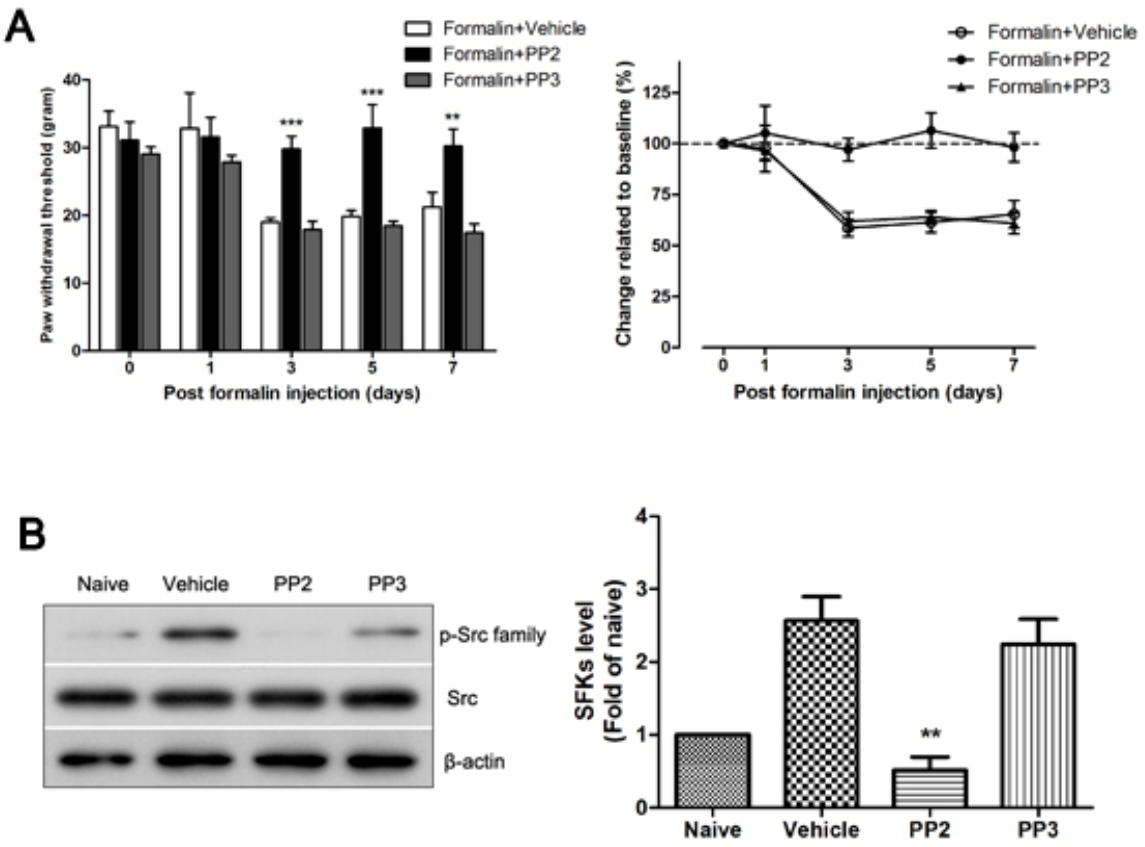

Figure 4 Effects of the SFK inhibitor PP2 delivered intrathecally on formalin-evoked persistent hyperalgesia. **A**, The mechanical hypersensitivity and percentage change from the baseline threshold were determined at days 1, 3, 5, and 7 after formalin injection, n = 6. **B**, Western blot analysis reveals inhibition of SFK activation by PP2. Quantification of Western blot data is shown at right. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the vehicle control, n = 4.

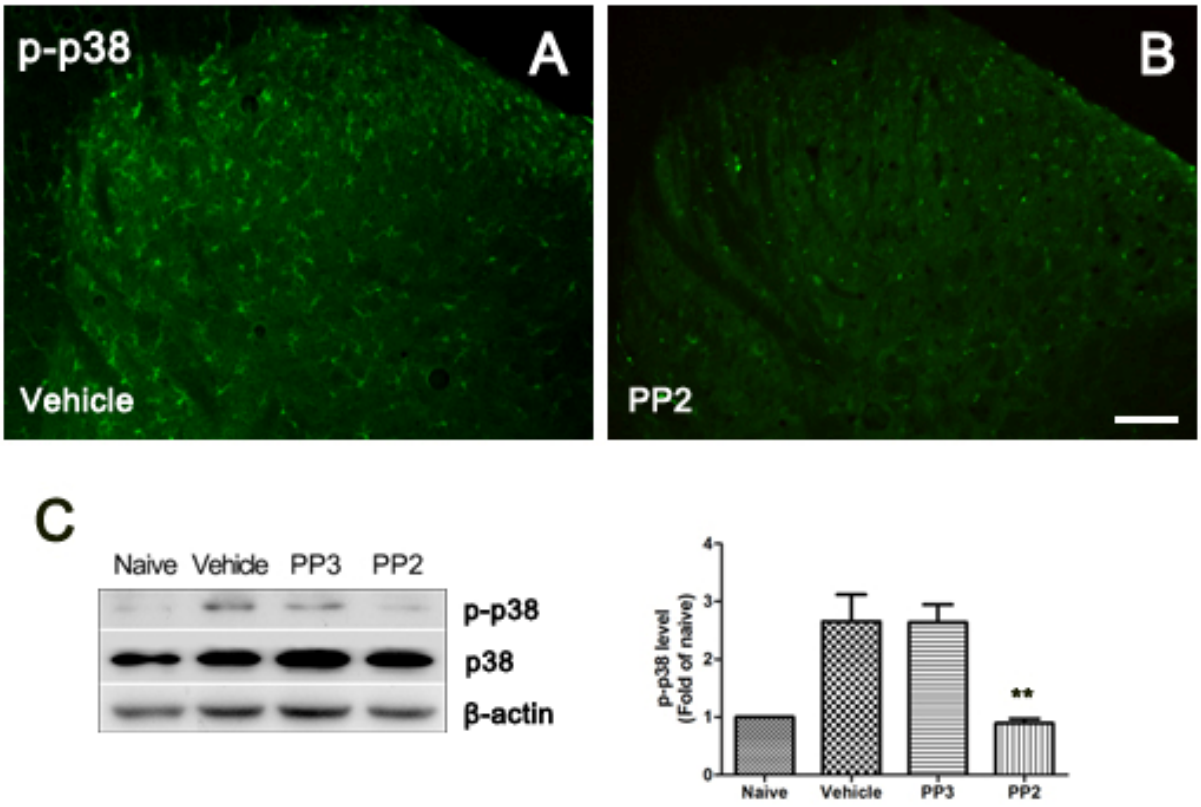

Figure 5 Effects of the SFK inhibitor PP2 delivered intrathecally on formalin-induced p38 MAPK activation in the dorsal horn. **A-C**, p-p38 immunostaining in the dorsal horn in the vehicle, PP3 and PP2 groups at day 3 after formalin injection. Scale bar: 50 μm. **D**, Western blot analysis indicated that PP2 suppressed the activation of p38 MAPK in the ipsilateral spinal cord at day 7. $^{**}P < 0.01$ compared with the vehicle control, n = 4.