Pain Physician 2018; 21:E555-E564 • ISSN 2150-1149

Animal Study

Contribution of Spinal PKCγ Expression to Short- and Long-lasting Pain Behaviors in Formalin-induced Inflamed Mice

Ya-Qun Zhao, MD1, Jun-bin Yin, MD2,3, Huang-Hui Wu, MD4, Tan Ding, MD5, Yong Wang, MD1, Jin-Chuan Liang, MD1, Xiao-Ju Guo, MD1, Ke Tang, MD1, Dong-Sheng Chen, MD4, and Guo-Zhong Chen, PhD4

From: [1]Department of Neurosurgery, the 309th Hospital of PLA, Beijing, PR China; [2]Department of Neurology, the 456th hospital of PLA, Jinan, PR China; [3]Department of Human Anatomy & K.K. Leung Brain Research Centre, Preclinical School of Medicine, the Fourth Military Medical University, Xi'an, PR China; [4]Department of Anesthesiology, Fuzhou General Hospital of PLA, Fuzhou, PR China; [5]Department of Orthopedics, Xijing Hospital, the Fourth Military Medical University, Xi'an, PR China

Address Correspondence:
Guo-Zhong Chen, MD
Department of Anesthesiology
Fuzhou General Hospital of PLA
No. 156, North of West Erhuan Rd
Fuzhou 350025, PR China
E-mail: fzzymzk@126.com

Disclaimer: This work is partly supported by the Natural Science Foundation of China (81701091), the Key Project of Nanjing Military Region (15ZD038, 15DX024), the Natural Science FOUNDATION of Fujian Province (2016J01476, 2016J01588, 2016Y0072), and an intramural grant from the Fourth Military Medical University (2015D06) and Fuzhou General Hospital (2015Q02). J-B Yin is also supported by the China Scholar Council. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Manuscript received: 04-12-2017
Revised manuscript received: 01-02-2018
Accepted for publication: 01-26-2018

Free full manuscript: www.painphysicianjournal.com

Background: Over-expression of spinal protein kinase C γ (PKCγ) contributes to the induction of persistent bilateral hyperalgesia following inflammatory injury, yet the role of spinal PKCγ in short- and long-lasting pain behavior is poorly understood.

Objective: This study aimed to characterize the contribution of spinal PKCγ to spontaneous pain and long-lasting bilateral hyperalgesia in formalin-induced inflamed mice using pharmacological inhibition.

Study Design: Laboratory animal study.

Setting: The study was performed in the Department of Human Anatomy and K.K. Leung Brain Research Centre, Preclinical School of Medicine, the Fourth Military Medical University (Xi'an, China) and the Department of Anesthesiology, Fuzhou General Hospital (Fuzhou, China).

Methods: Male mice were unilaterally intraplantarly injected with formalin to induce inflammatory pain. Spontaneous pain behaviors, including flinches and lickings, were recorded by off-line video during the first hour post-injection and counted. Using von Frey tests, long-lasting bilateral mechanical paw withdrawal thresholds were determined before injection and at indicated time points thereafter. Temporal expression of spinal PKCγ was observed by immunohistochemical staining. For pharmacological inhibition, mice were treated daily with intrathecal Tat carrier or selective PKCγ inhibitor KIG31-1, from 1 hour prior to 10 days after formalin injection. Spontaneous pain behaviors and long-lasting bilateral mechanical hyperalgesia were assessed. Spinal PKCγ expression was also observed by using immunohistochemical staining and western blot.

Results: The number of PKCγ-immunoreactive (ir) spinal neurons was significantly higher at 10 days, but not 2 hours, after formalin intraplantar injection, and accompanied by long-lasting bilateral hyperalgesia. Furthermore, long-lasting bilateral hyperalgesia could be reversed by pharmacological inhibition of over-expressed spinal PKCγ; however, pretreating with intrathecal KIG31-1 showed no antinociceptive effects on short-term spontaneous pain behaviors.

Limitations: All results were obtained from the mice and no PKCγ inhibitors were available through clinical practice. Therefore, it remains difficult to draw definitive connections between animal research and human application.

Conclusion: Our findings suggest that spinal PKCγ plays a predominant role in long-lasting bilateral hyperalgesia, but not in the spontaneous pain behaviors induced by formalin.

Key words: Formalin, spontaneous pain, mechanical hyperalgesia, protein kinase C gamma, KIG31-1, mice

Pain Physician 2018: 21:E555-E564

Subcutaneous (subq) injection of formalin is commonly used as a classic model of acute inflammatory pain observable by primary spontaneous nocifensive behaviors including flinches and lickings (1-3). According to our previous research, a secondary and long-lasting mechanical allodynic and hyperalgesic response can be observed not only on the ipsilateral but also on the contralateral hind paw. Since sensory receptors in the bilateral area of secondary mechanical allodynia and hyperalgesia are unaffected, these sensory changes might be caused by central sensitization in the spinal cord and brain nucleus of the initial intense nociceptive discharge that follows the formalin injection (4-6). Various studies suggest that the release of immune substances and proinflammatory cytokines from spinal glia (7,8), and the bilateral signaling via commissural interneurons (9), might, in part, induce the mirror-image pain. Furthermore, the long-lasting increase in excitability of spinal neurons also depends on the activation of microglial, calcium/calmodulin-dependent kinase II; and several second messenger cascades including protein kinase C (PKC) signal transduction pathways (1,10,11).

Spinal PKC gamma (PKCγ) is restrictedly expressed in a subpopulation of interneurons within the inner lamina II of the spinal dorsal horn and plays an important role in the processing of tactile inputs under both physiological and pathological conditions (12,13); its over-expression contributes to the induction of persistent bilateral hyperalgesia following inflammatory injury. Long-lasting mechanical hyperalgesia accompanied by more than 75% up-regulation of PKCγ expression in the bilateral spinal cord can be observed in a complete Freund's adjuvant (CFA)-induced chronic inflammatory pain rat model (14). Knocking out PKCγin mice that have undergone partial sciatic nerve section almost completely fails to induce neuropathic pain along with spinal neurochemical change and inflammatory response; however, lacking PKCγ does not inhibit a normal response to acute pain stimuli (12). More recently, evidence points toward the key role of spinal PKCγ+ neurons in the modulation of the "gate control" circuit during the bilateral hyperalgesic process. Previous data shows the convergence of glycinergic inhibitory and excitatory Aβ-fiber inputs onto PKCγ+ neurons in the superficial dorsal horn; this convergence forms a feed-forward inhibitory circuit that prevents nociceptive input. This feed-forward inhibition is suppressed following peripheral injury or glycine blockage, leading to induction of long-lasting mechanical allodynia and hyperalgesia (15,16). The above evidence indicates that inhibition of spinal PKCγ-immunoreactive (ir) neurons is a possible target in the nociceptive transformation. In this study, we use pharmacological inhibition in formalin-induced inflamed mice to characterize the contribution of spinal PKCγ to spontaneous pain and long-lasting bilateral hyperalgesia.

Methods

Animals

Male C57BL/6 mice (8 weeks old) were housed in a temperature-controlled environment on a 12-hour light/dark cycle with free access to food and water. All experimental procedures received prior approval from the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi'an, China) and Fuzhou General Hospital (Fuzhou, China), We followed ethical guidelines for investigating experimental pain in conscious animals.

Experimental Design

According to our previous studies (3,4,17), the behavioral features of mice receiving intraplantar saline were similar to those of naïve mice; thus, the behavioral data obtained from the naïve mice were not included in this study. A two-part experiment was designed to characterize the role of spinal PKCγ in the short- and long-lasting pain behavior of formalin-induced inflamed mice.

The first part aimed to observe the temporal relation of spinal PKCγ expression to formalin-induced spontaneous pain behaviors, including flinches and lickings, together with long-lasting bilateral mechanical hyperalgesia. After a 5-day acclimation period, mice were randomly assigned to one of the following groups: (1) mice receiving subq injections with 25 μL of saline; or (2) mice receiving subq injections with 25 μL of 5% formalin. For 1 hour after receiving subq injections, all of the animals from the 2 groups were video- and audio-recorded for later off-line analysis.

The second part aimed to identify the role of spinal PKCγ in formalin-induced spontaneous pain behaviors and long-lasting bilateral mechanical hyperalgesia using pharmacological inhibition. After a 5-day acclimation period, the animals were randomly assigned to one of the following groups: (1) mice receiving intrathecal (IT)injections of 5 μL of Tat carrier once daily, from 1

hour prior, to 10 days after, subq injections with 25 μL of 5% formalin (Forma+Tat group); (2) mice receiving IT injections of 5 μL of 100 pmol KIG31-1 once daily from 1 hour prior, to 10 days after, subq injections with 25 μL of 5% formalin (Forma+KIG31-1 group).

Assessment of Spontaneous Pain Behaviors

The formalin test was used to induce flinching or licking of the injected hind paw, as described in our previous study (3). All behavioral observations were performed in a low-illuminated sound-proof room. A sound-attenuated clear perspex testing cage (25*25*40 cm) was fitted with a reverse video camera to record video for off-line behavioral analysis. After the mice's acclimation to the testing chamber for 20 minutes, formalin solution (dissolved in saline) was intraplantarly injected on the right hind paw using a microsyringe attached to a 30-gauge needle. After formalin injection, mice were returned to the observing cage and the video- and audio-recordings were taken for 1 hour. A trained observer blinded to the injection conducted the behavioral analysis of the video recordings. The pain behaviors were manually recorded with a stopwatch by retrieving spontaneous flinches or lickings of the injected hind paw from the recorded videos.

Measurement of Behavior to Mechanical Stimuli

Mice were habituated to the testing environment for 3 days before baseline testing, and were then placed under inverted plastic boxes (7*7*10 cm) on an elevated mesh floor and allowed to habituate for 30 minutes before the threshold testing. A logarithmic series of 8 calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Kiel, WI) were applied to the lateral edge on the plantar surface of the injected paw, as well as on the plantar surface of the contralateral paw, to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. A von Frey filament was applied 10 times (3 seconds for each stimulus) to each testing area. The bending force of the von Frey filament able to evoke a 50% occurrence of the paw withdrawal reflex was expressed as the paw withdrawal threshold (PWT). The stimulus was stopped if the threshold exceeded 10.0 g (cutoff value). Baseline values were assessed prior to formalin injection. Subsequent behavioral tests were performed at 1-, 3-, 7-, and 10-days post-injection. The threshold was defined as the minimum pressure required to elicit a withdrawal reflex. The percent change in PWT was determined for the injected paw relative to the contralateral paw, according to the formula: (ipsilateral PWT – contralateral PWT)/contralateral PWT. This index was used as a measure of mechanical hyperalgesia associated with the injected hind paw relative to the contralateral hind paw. Positive values indicated a state of hypoalgesia, whereas negative values indicated a state of hyperalgesia associated with the injected hind paw. All tests were performed in a double-blind manner.

Intrathecal Injection of KIG31-1

Mice were shaved at the back of the lumbar area where IT injections were given. Tat carrier or KIG31-1 was injected into the spinal canal using a 10 μL microsyringe (Hamilton Company, Reno, NV) attached to a 32-gauge needle. KIG31-1 was obtained from Kai Pharmaceuticals (San Francisco, CA). It is conjugated to Tat, a peptide carrier, via a cysteine-cysteine bond at its N-terminus. KIG31-1 competes with activated PKCγ for binding to the isoenzyme-specific docking proteins, receptors for activated C kinase. This strategy prevents PKCγ translocation in an isozyme-specific manner (18,19). Linking of KIG31-1 to Tat enables efficient transfer of the peptide into cells (20).

Immunohistochemical Staining

After deep anesthesia induced by pentobarbital (100 mg/kg, i.p.), and perfusion with 30 mL of 0.9% saline followed by 100 mL of 0.1 M phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde, the lumbar spinal cord segments were removed and post-fixed in the same agent for 4 hours and then immersed in 30% sucrose in 0.1 M PB (pH 7.4). Transverse spinal sections were cut to 25 μm thickness on a cryostat (Leica CM1800, Heidelberg, Germany) at -20°C and sections were collected serially into dishes containing 0.01 M phosphate-buffered saline (PBS, pH 7.4).

Immunohistochemical staining for PKCγ was performed using the fluorescent method. The sections were rinsed in 0.01 M PBS 3 times (10 minutes each), blocked with 10% donkey serum in 0.01 M PBS containing 0.3% Triton X-100 for 0.5 hours at room temperature (RT), and then used for immunohistochemical staining. The sections were incubated overnight at RT with rabbit anti-PKCγ antiserum (1:500; sc-211; Santa Cruz Biotechnology, Santa Cruz, CA) in 0.01 M PBS containing 5% normal donkey serum, 0.03% Triton X-100, 0.05% NaN3, and 0.25% carrageenan (PBS-NDS,

pH 7.4), followed by incubation with Cy3-conjugated donkey anti-rabbit secondary antibody (1:500; AP182C; Millipore, Billerica, MA) diluted in PBS-NDS for 4 hours. Sections were washed completely with 0.01 M PBS between each step. Finally, all sections were mounted onto gelatin-coated glass slides, air dried, and coverslipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylenediamine (anti-fading agent) in 0.01 M PBS, and then observed using a fluorescence microscope.

Western Blot

Mice were sacrificed under deep anesthesia by pentobarbital (100 mg/kg, i.p.) and the L4-5 spinal cord was quickly removed. Briefly, the spinal segment was cut into left and right halves from the midline, and the dorsal horns of both halves were collected. The selected region was homogenized with a hand-held pestle in a sodium dodecyl sulfate (SDS) sample buffer (60 μL/mg tissue) containing proteinase inhibitors. The electrophoresis samples were heated at 100°C for 5 minutes and loaded onto 10% SDS-polyacrylamide gels with standard Laemmli solutions (Bio-Rad Laboratories, Hercules, CA). After the proteins were electroblotted onto a polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore, Billerica, MA), the membranes were placed in a blocking solution containing Tris-buffered saline with 0.02% Tween (TBS-T) and 5% fetal bovine serum, and incubated for 60 minutes under gentle agitation at RT, then overnight at 4°C with rabbit anti-PKCγ antiserum (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-β-actin antibody (1:5000; Sigma, St Louis, MO). Bound primary antibodies were detected by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10000; Amersham Pharmacia Biotech Inc., Piscataway, NJ) for 2 hours under gentle agitation at RT. Between each step, the immunoblots were rinsed 3 times with TBS-T for 10 minutes each. The protein blots' densities were detected and analyzed in the Bio-Rad ChemiDocTM Imaging System (Bio-Rad Laboratories Ltd, Hercules, CA).

Statistical Analysis

The results are expressed as mean value ± standard deviation (SD). The area under curves (AUCs) for individual mice showing formalin-induced flinches or licking responses were pooled and analyzed by Student's t tests. Mechanical hyperalgesia and expressions of spinal PKCγ were also analyzed by Student's t tests. Inhomogeneous variance data was analyzed using the Kruskal-Wallis test. All data analyses were performed using GraphPad Prism version 5.01 for Windows (Graph Pad Software, San Diego, California). A P value of less than 0.05 was considered statistically significant.

Results

Spontaneous Pain Behaviors and Mechanical Hypo- or Hypersensitivity Induced by Intraplantar Formalin

Subq formalin injection with formalin produced biphasic flinches and lickings of the injected paw, the first transient phase lasting for the first 10 minutes and the second phase lasting from the 15- to the 60-minute mark (Fig. 1A). As summarized by the AUC values of flinches and lickings, the algogenic profile of subq formalin with formalin can be seen in both the first and second phases (Fig. 1B; Student's t test; $P < 0.001$ in each).

There were no significant differences in baseline PWTs to mechanical stimuli between the ipsilateral and contralateral hind paws. One day after receiving subq formalin injection with formalin, a significant increase in PWTs (relative to baseline values) on the ipsilateral hind paw was observed (Fig. 2A; $P < 0.001$); this hypoalgesic status continued to 3 days post-injection (Fig. 2A; $P < 0.05$). After 3 days, however, a hyperalgesic status was observed, indicated by a decrease in PWTs relative to baseline values (Fig. 2A; $P < 0.05$). On the contralateral hind paw, a persistent significant decrease of PWTs compared with baseline PWTs was observed from 1 day to 10 days post-injection. Furthermore, from 3 days to 10 days post-injection, there was a long-lasting bilateral mechanical hyperalgesia indicated by similar PWTs for the ipsilateral and the contralateral hind paws (Fig. 2B; $P < 0.001$).

Increase of Spinal PKCγ Expression During Long-lasting but Not Short-term Inflammatory Pain

The distribution and expression of spinal PKCγ were checked by using immunohistochemistry and western blot at 2 hours and at 10 days after formalin injection, respectively. The immunoreactivity of PKCγ, including terminals and neurons, was observed in the superficial dorsal horn, especially restricted distribution of spinal PKCγ in the lamina IIi (Fig. 3). There were no significant differences in expression of spinal PKCγ between the ipsilateral and the contralateral dorsal horns at 2 hours after formalin injection (Figs. 3A1, A2, B1, B2, C1; $P > 0.05$). Meanwhile, similar levels of expression of spinal

Fig. 1. ***Spontaneous pain behaviors of s.c. formalin-induced flinches and lickings of the injected hind paw.***
Spontaneous flinches and lickings per 5 minute interval during 60 minute period after subcutaneous (subq) formalin or saline injection are shown in A, respectively. The AUCs were calculated for statistical analysis of the first and second phases, as shown in B. n = 6, each group. *$P < 0.05$.

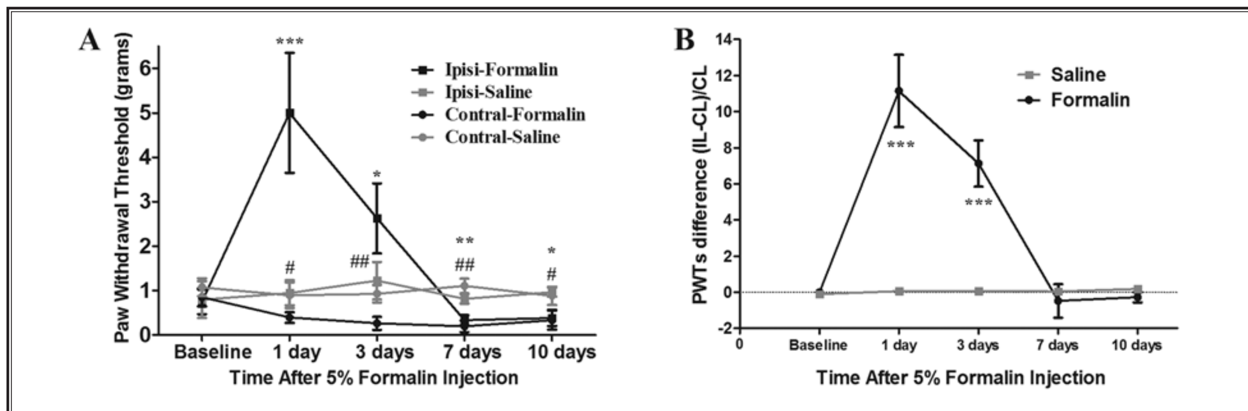

Fig. 2. ***A 10-day change in mechanical paw withdrawal thresholds induced by subq formalin or saline injection.***
Paw withdrawal thresholds for ipsilateral and contralateral hind paws after 10 days of subq formalin or saline injection are shown in A. Injection of formalin induced both mechanical hyposensitivity and hypersensitivity in the ipsilateral hind paw from 1 day to 10 days after injection, and mechanical hypersensitivity in the contralateral hind paw. The differences in paw withdrawal thresholds between formalin- and saline-injected groups are shown in B. Difference in paw withdrawal thresholds = (ipsilateral PWT – contralateral PWT) / contralateral PWT. n = 6, each group. Formalin-injected ipsilateral compared with saline-injected ipsilateral hind paw, *$P < 0.05$, **$P < 0.01$, ***$P < 0.01$. Formalin-injected contralateral compared with saline-injected contralateral hind paw, #$P < 0.05$, ##$P < 0.01$.

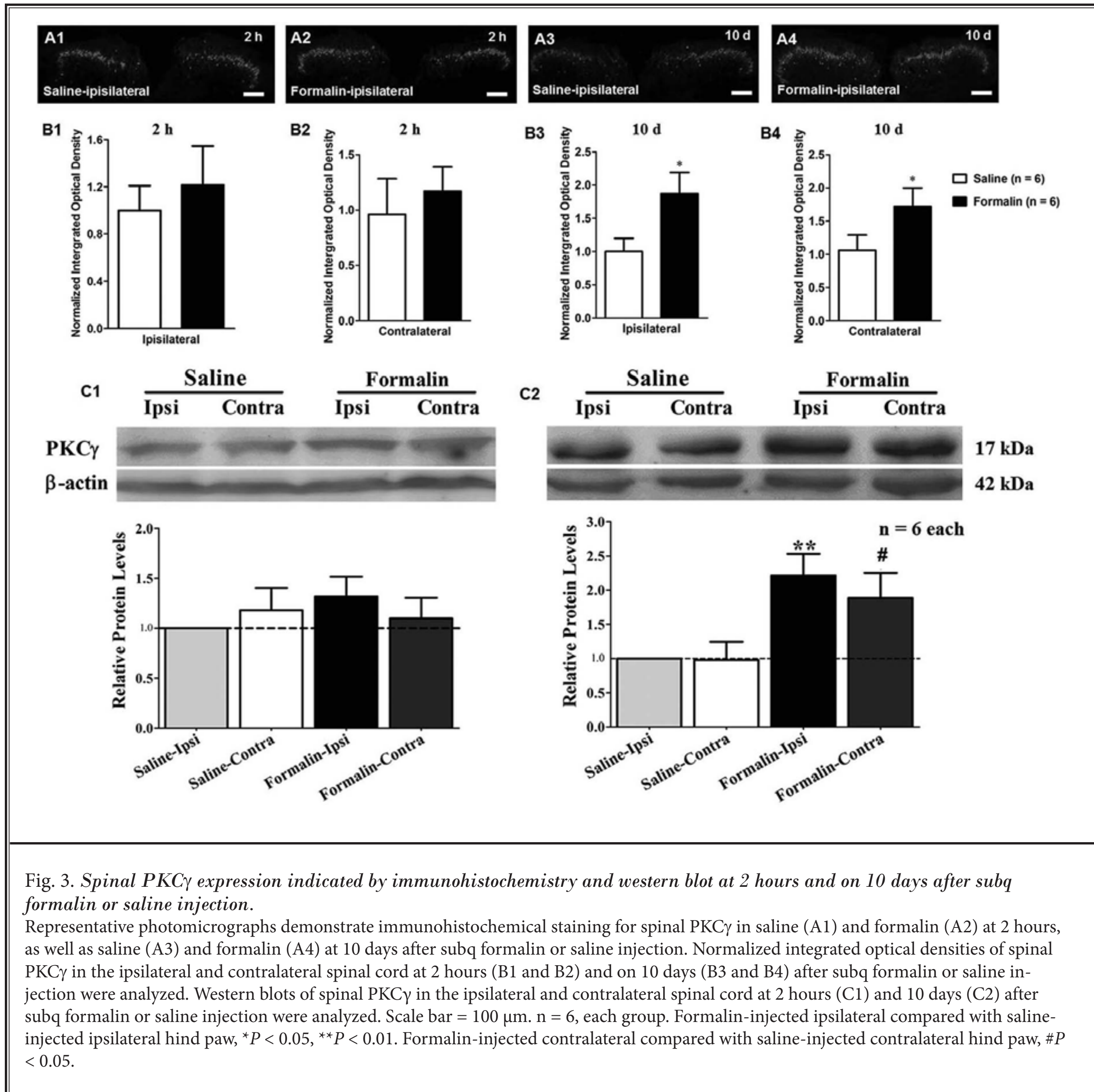

Fig. 3. *Spinal PKCγ expression indicated by immunohistochemistry and western blot at 2 hours and on 10 days after subq formalin or saline injection.*

Representative photomicrographs demonstrate immunohistochemical staining for spinal PKCγ in saline (A1) and formalin (A2) at 2 hours, as well as saline (A3) and formalin (A4) at 10 days after subq formalin or saline injection. Normalized integrated optical densities of spinal PKCγ in the ipsilateral and contralateral spinal cord at 2 hours (B1 and B2) and on 10 days (B3 and B4) after subq formalin or saline injection were analyzed. Western blots of spinal PKCγ in the ipsilateral and contralateral spinal cord at 2 hours (C1) and 10 days (C2) after subq formalin or saline injection were analyzed. Scale bar = 100 μm. n = 6, each group. Formalin-injected ipsilateral compared with saline-injected ipsilateral hind paw, $^*P < 0.05$, $^{**}P < 0.01$. Formalin-injected contralateral compared with saline-injected contralateral hind paw, $\#P < 0.05$.

PKCγ in the ipsilateral dorsal horn were observed at 2 hours after either formalin injection or saline injection ($P > 0.05$), while that in both the ipsilateral and contralateral dorsal horns was significantly higher at 10 days (Fig. 3A3, A4, B3, B4, C2; $P < 0.05$) and that was without significant difference between the 2 sides of the dorsal horn ($P > 0.05$). These results indicate that spinal PKCγ was involved in the formalin-induced long-lasting, but not short-term, inflammatory pain.

Prevention of Formalin-induced Long-lasting but Not Short-term Inflammatory Pain by Selectively Inhibiting PKCγ Activation

After receiving consecutive IT injections of 5 μL of 100 pmol KIG31-1 once daily from 1 hour prior, to 10 days after, subq injections with 25 μL of 5% formalin, spontaneous and long-lasting pain behaviors were observed. Consistent with the temporal changes in spinal PKCγ expression, pharmacological inhibition

with KIG31-1 did not inhibit the flinching and licking behaviors either in the first or the second phase after formalin injection (Fig. 4A). There was no significant difference in the AUCs of flinches and lickings during the indicated time course (Fig. 4B; $P > 0.05$ in each), which indicated no analgesic effect of inhibiting spinal PKCγ expression on spontaneous pain induced by formalin injection. There was no improvement of the ipsilateral hypoalgesia at 1 day and 3 days after formalin injection? (Fig. 5A; $P > 0.05$ in each); however, the hyperalgesic status in the contralateral hind paw from 1 day to 10 days, and that in the ipsilateral hind paw from 7 days to 10 days, was reversed (Fig. 5B; $P < 0.05$ in each). These results further confirmed that spinal PKCγ was involved in the formalin-induced long-lasting, but not short-term, inflammatory pain.

Discussion

Consistent with the key role of spinal PKCγ in the maintenance of persistent pain, our study showed that long-lasting bilateral mechanical hyperalgesia is accompanied by over-expression of PKCγ in the bilateral dorsal horn 10 days, but not 2 hours, after receiving subq formalin injection. Moreover, this long-lasting hyperalgesic status, rather than spontaneous pain behaviors, could be reserved by consecutive intrathecal administration of KIG31-1, which selectively inhibits spinal PKCγ activation. These findings indicate that spinal PKCγ is involved in the development of long-lasting, but not short-term, inflammatory pain in formalin-induced inflamed mice.

Chronic pain is a persistent state of peripheral and/or central sensitization that occurs after acute tissue injury. Chronic pain presents as an exaggerated response to nociceptive stimuli, pain in response to normally innocuous stimuli (21,22). Although our natural mental perception of, and physical response to, acute pain serve an indispensable protective function, the long-lasting and overreacted changes in nociceptive processing, transmission, and perception caused by such sensitization are harmful. Previous research has shown that peripheral nerve injury produces a persistently neuropathic pain state in which pain is exaggerated and can be produced by innocuous stimuli in wild-type animals (12). Interestingly, mice with knocked-out or inhibited spinal PKCγ expression display normal responses to acute nociceptive stimuli, but they almost completely fail to develop chronic pain (12,23-25). This evidence indicates that spinal PKCγ is a promising target for the prevention and therapy of chronic pain.

Previous morphological studies have confirmed that PKCγ is concentrated in the spinal interneurons located in the inner part of lamina II (IIi) of the dorsal horn and can be activated by tactile inputs, and that these PKCγ interneurons can participate in local circuits connecting tactile inputs to nociceptive output neurons in lamina I (13). In marked contrast to the results from innocuous stimulation, in which double-labeled PKCγ/FOS interneurons were observed in lamina IIi, noxious stimulation by formalin injection into the hind paw failed to produce double-labeled PKCγ/FOS interneurons (26). Triple immunofluorescence labeling revealed co-localization of the NMDAR and 5-hydroxytryptamine (5-HT) 2B receptors in PKCγ-expressing perikarya in lamina II neurons (27). The 5-HT2B receptor-induced proliferative response of the interstitial cells of Cajal ICC is through phospholipase C, Ca^{2+}, and PKCγ, implicating this PKC isoform in the regulation of cellular proliferation (28). PKCγ is involved in the regulation of the 5-HT2A mRNA receptor and binding sites in response to agonist treatment (29). More importantly, it also reflects adaptive changes in the inhibitory and/or excitatory descending pathways in response to inflammation-facilitated spinal nociception. Our previous studies have shown that spinal expressions of 5-HT transporter are involved in producing long-lasting mechanical hyperalgesia; 5-HT transporter transports serotonin from synaptic spaces into presynaptic neurons and thus, plays an essential role in determining the duration and intensity of 5-HT communication with its receptors (3). Activation of PKCγ by Tetanus toxin results in a loss of transport capacity and serotonin transporter phosphorylation; both effects are abolished by coapplication of the specific PKCγ inhibitor bisindolylmaleimide-1 (30). The evidence also shows repeated inflammation-induced expression of PKCγ in the membrane of spinal dorsal horn neurons and increased PKCγ level in the cytosol (31). With hyperglycemia and body weight loss, streptozotocin mice exhibited orofacial thermal hyperalgesia, along with increased PKCγ expression in trigeminal spinal nucleus (32). Early transcutaneous electrical nerve stimulation reduced mechanical and thermal hyperalgesia in the neuropathic pain model by inhibiting PKCγ expression (33).

Not only does the sensitization cause allodynia and/or hyperalgesia on the ipsilateral lesioned side, but it also affects the contralateral nonlesioned structures; this is referred to as mirror image pain. The pathogenesis of mirror image pain is still poorly understood, but transneuronal signaling via interneuronal mechanisms between the ipsilateral and contralateral

A

Flinch

Forma+Tat (n=6)

Forma+KIG31-1 (n=6)

Flinches/5min

Time(min)

Lick

Duration (sec/5min)

Time (min)

B

1st phase

2nd phase

AUC

Flinch

Lick

Fig. 4. *Effect of intrathecal (i.t.) KIG31-1 on spontaneous pain behaviors induced by subq formalin.*
Spontaneous flinches and lickings per every 5 minute interval during 60 minute period after subq formalin injection in Tat carrier vs KIG31-1 groups are shown in A, respectively . The AUCs were calculated for statistical analysis of the first and second phases, as shown in B. n = 6, each group.

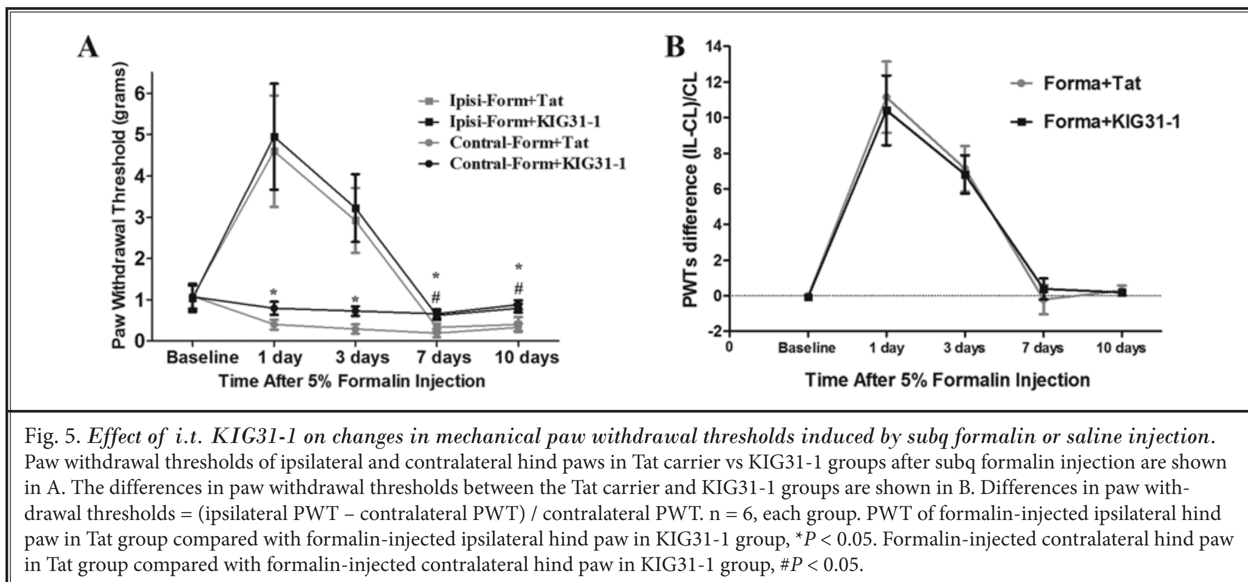

Fig. 5. *Effect of i.t. KIG31-1 on changes in mechanical paw withdrawal thresholds induced by subq formalin or saline injection.*
Paw withdrawal thresholds of ipsilateral and contralateral hind paws in Tat carrier vs KIG31-1 groups after subq formalin injection are shown in A. The differences in paw withdrawal thresholds between the Tat carrier and KIG31-1 groups are shown in B. Differences in paw withdrawal thresholds = (ipsilateral PWT – contralateral PWT) / contralateral PWT. n = 6, each group. PWT of formalin-injected ipsilateral hind paw in Tat group compared with formalin-injected ipsilateral hind paw in KIG31-1 group, *$P < 0.05$. Formalin-injected contralateral hind paw in Tat group compared with formalin-injected contralateral hind paw in KIG31-1 group, #$P < 0.05$.

reaction have been confirmed (34,35). Previous study showed that the spinal PKC, including PKCγ, is likely to be involved in the central process of mirror-image hypersensitivity but not primary mechanical hypersensitivity in a bee venom chemical injury rat model (36,37). Our results showed similar expressions of spinal PKCγ between the ipsilateral and contralateral dorsal horns at 2 hours after formalin injection, as well as similar expressions of spinal PKCγ in the ipsilateral dorsal horn between the formalin and saline groups, indicating no role of spinal PKCγ in spontaneous pain behaviors. However, over-expression of PKCγ in the bilateral dorsal horn 10 days after formalin injection, accompanied by long-lasting mechanical hyperalgesia, was able to be reversed by selectively inhibiting spinal PKCγ activation using intrathecal KIG31-1 injection. These data positively suggest the essential contribution of spinal PKCγ activation to long-lasting mechanical hyperalgesia. Interestingly, we also reported a dual sensory change over days 1 through 3 on the ipsilateral hind paw. As we discussed in the previous study, the hypoalgesic status might be derived from inflammatory edema and sensory receptor damage.

In summary, the current results provide further evidence of the essential contribution of spinal PKCγ activation to long-lasting mechanical hyperalgesia, but not short-term spontaneous pain behavior, in formalin-induced inflamed mice.

Acknowledgement

Y-Q Zhao, J-B Yin, and H-H Wu contributed equally to this manuscript as co-first authors. Y-Q Zhao, D-S Chen and G-Z Chen contributed equally in designing this work as co-corresponding authors.

Funding/Support: This work is partly supported by the Natural Science Foundation of China (81701091), the Key Project of Nanjing Military Region (15ZD038, 15DX024), the Natural Science Foundation of Fujian Province (2016J01476, 2016J01588, 2016Y0072), and an intramural grant from the Fourth Military Medical University (2015D06) and Fuzhou General Hospital (2015Q02). J-B Yin is also supported by the China Scholar Council. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Fu KY, Light AR, Maixner W. Long-lasting inflammation and long-term hyperalgesia after subcutaneous formalin injection into the rat hindpaw. *J Pain* 2001; 2:2-11.
2. Godínez-Chaparro B, Barragán-Iglesias P, Castañeda-Corral G, Rocha-González HI, Granados-Soto V. Role of peripheral 5-HT(4), 5-HT(6), and 5-HT(7) receptors in development and maintenance of secondary mechanical allodynia and hyperalgesia. *Pain* 2011; 152:687-697.
3. Yin JB, Zhou KC, Wu HH, Hu W, Ding T, Zhang T, Wang LY, Kou JP, Kaye AD, Wang W. Analgesic effects of danggui-shaoyao-san on various "phenotypes" of nociception and inflammation in a formalin pain model. *Mol Neurobiol* 2016; 10:6835-6848.
4. Bai L, Wang W, Dong YL, Wang W, Huang J, Wang XY, Wang LY, Li YQ, Wu SX. Attenuation of mouse somatic and emotional inflammatory pain by hydralazine through scavenging acrolein and inhibiting neuronal activation. *Pain Physician* 2012; 15:311-326.
5. Martínez-Rojas VA, Barragán-Iglesias P, Rocha-González HI, Murbartián J, Granados-Soto V. Role of TRPV1 and ASIC3 in formalin-induced secondary allodynia and hyperalgesia. *Pharmacol Rep* 2014; 66:964-971.
6. Yin JB, Wu HH, Dong YL, Zhang T, Wang J, Zhang Y, Wei YY, Lu YC, Wu SX, Wang W, Li YQ. Neurochemical properties of BDNF-containing neurons projecting to rostral ventromedial medulla in the ventrolateral periaqueductal gray. *Front Neural Circuits* 2014; 8:137.
7. Twining CM, Sloane EM, Milligan ED, Chacur M, Martin D, Poole S, Marsh H, Maier SF, Watkins LR. Peri-sciatic proinflammatory cytokines, reactive oxygen species, and complement induce mirror-image neuropathic pain in rats. *Pain* 2004; 110:299-309.
8. Watkins LR, Milligan ED, Maier SF. Spinal cord glia: New players in pain. *Pain* 2001; 93:201-205.
9. Koltzenburg M, Wall PD, McMahon SB. Does the right side know what the left is doing? *Trends Neurosci* 1999; 22:122-127.
10. Fu KY, Light AR, Maixner W. Relationship between nociceptor activity, peripheral edema, spinal microglial activation and long-term hyperalgesia induced by formalin. *Neuroscience* 2000; 101:1127-1135.
11. Fu KY, Light AR, Matsushima GK, Maixner W. Microglial reactions after subcutaneous formalin injection into the rat hind paw. *Brain Res* 1999; 825:59-67.
12. Malmberg AB, Chen C, Tonegawa S, Basbaum AI. Preserved acute pain and reduced neuropathic pain in mice

lacking PKCgamma. *Science* 1997; 278:279-283.

13. Polgar E, Fowler JH, McGill MM, Todd AJ. The types of neuron which contain protein kinase C gamma in rat spinal cord. *Brain Res* 1999; 833:71-80.
14. Martin WJ, Liu H, Wang H, Malmberg AB, Basbaum AI. Inflammation-induced up-regulation of protein kinase Cgamma immunoreactivity in rat spinal cord correlates with enhanced nociceptive processing. *Neuroscience* 1999; 88:1267-1274.
15. Miraucourt LS, Dallel R, Voisin DL. Glycine inhibitory dysfunction turns touch into pain through PKCgamma interneurons. *PLoS One* 2007; 2:e1116.
16. Lu Y, Dong H, Gao Y, Gong Y, Ren Y, Gu N, Zhou S, Xia N, Sun YY, Ji RR, Xiong L. A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia. *J Clin Invest 2013;* 123:4050-4062.
17. Sun YH, Dong YL, Wang YT, Zhao GL, Lu GJ, Yang J, Wu SX, Gu ZX, Wang W. Synergistic analgesia of duloxetine and celecoxib in the mouse formalin test: A combination analysis. *PLoS One* 2013; 8:e76603.
18. Churchill EN, Qvit N, Mochly-Rosen D. Rationally designed peptide regulators of protein kinase C. *Trends Endocrinol Metab* 2009; 20:25-33.
19. Pham-Dang N, Descheemaeker A, Dallel R, Artola A. Activation of medullary dorsal horn gamma isoform of protein kinase C interneurons is essential to the development of both static and dynamic facial mechanical allodynia. *Eur J Neurosci* 2016; 43:802-810.
20. Chen L, Wright LR, Chen CH, Oliver SF, Wender PA, Mochly-Rosen D. Molecular transporters for peptides: Delivery of a cardioprotective epsilonPKC agonist peptide into cells and intact ischemic heart using a transport system, R(7). *Chem Biol* 2001; 8:1123-1129.
21. Woolf CJ. Central sensitization: Implications for the diagnosis and treatment of pain. *Pain* 2011; 152:S2-S15.
22. Luongo L, Malcangio M, Salvemini D, Starowicz K. Chronic pain: New insights in molecular and cellular mechanisms. *Biomed Res Int* 2015; 2015:676725.
23. Wu B, Wang S, Qin G, Xie J, Tan G, Zhou J, Chen L. Protein kinase C gamma contributes to central sensitization in a rat model of chronic migraine. *J Mol Neurosci* 2017; 63:131-141.
24. Hang LH, Li SN, Dan X, Shu WW, Luo H, Shao DH. Involvement of spinal CCR5/PKCgamma Signaling pathway in the maintenance of cancer-induced bone pain. *Neurochem Res* 2017; 42:563-571.
25. Ko MH, Yang ML, Youn SC, Lan CT, Tseng TJ. Intact subepidermal nerve fibers mediate mechanical hypersensitivity via the activation of protein kinase C gamma in spared nerve injury. *Mol pain* 2016; 12.
26. Neumann S, Braz JM, Skinner K, Llewellyn-Smith IJ, Basbaum AI. Innocuous, not noxious, input activates PKCgamma interneurons of the spinal dorsal horn via myelinated afferent fibers. *J Neurosci* 2008; 28:7936-7944.
27. Aira Z, Buesa I, Garcia del Cano G, Bilbao J, Doñate F, Zimmermann M, Azkue JJ. Transient, 5-HT2B receptor-mediated facilitation in neuropathic pain: Up-regulation of PKCgamma and engagement of the NMDA receptor in dorsal horn neurons. *Pain* 2013; 154:1865-1877.
28. Wouters MM, Roeder JL, Tharayil VS, Stanich JE, Strege PR, Lei S, Bardsley MR, Ordog T, Gibbons SJ, Farrugia G. Protein kinase Cgamma mediates regulation of proliferation by the serotonin 5-hydroxytryptamine receptor 2B. *J Biol Chem* 2009; 284:21177-21184.
29. Anji A, Sullivan Hanley NR, Kumari M, Hensler JG. The role of protein kinase C in the regulation of serotonin-2A receptor expression. *J Neurochem* 2001; 77:589-597.
30. Najib A, Pelliccioni P, Gil C, Aguilera J. Serotonin transporter phosphorylation modulated by tetanus toxin. *FEBS Lett* 2000; 486:136-142.
31. Hu W, Zhang Y, Cai Q, Wang D, Hong Y. Blockade of 5-HT2A receptors at the site of inflammation inhibits activation of spinal dorsal horn neurons in rats. *Brain Res Bull* 2016; 124:85-94.
32. Xie HY, Xu F, Li Y, Zeng ZB, Zhang R, Xu HJ, Qian NS, Zhang YG. Increases in PKC gamma expression in trigeminal spinal nucleus is associated with orofacial thermal hyperalgesia in streptozotocin-induced diabetic mice. *J Chem Neuroanat* 2015; 63:13-19.
33. Matsuo H, Uchida K, Nakajima H, Guerrero AR, Watanabe S, Takeura N, Sugita D, Shimada S, Nakatsuka T, Baba H. Early transcutaneous electrical nerve stimulation reduces hyperalgesia and decreases activation of spinal glial cells in mice with neuropathic pain. *Pain* 2014; 155:1888-1901.
34. Jancalek R. Signaling mechanisms in mirror image pain pathogenesis. *Ann Neurosci* 2011; 18:123-127.
35. Fitzgerald M. The contralateral input to the dorsal horn of the spinal cord in the decerebrate spinal rat. *Brain Res* 1982; 236:275-287.
36. Li KC, Chen J. Differential roles of spinal protein kinases C and a in development of primary heat and mechanical hypersensitivity induced by subcutaneous bee venom chemical injury in the rat. *NeuroSignals* 2003; 12:292-301.
37. Chen J. Spinal processing of bee venom-induced pain and hyperalgesia. *Acta physiologica Sinica* 2008; 60:645-652.