



# NF- $\kappa$ B mediated CX3CL1 activation in the dorsal root ganglion contributes to the maintenance of neuropathic pain induced in adult male Sprague Dawley rats<sup>1</sup>

Chenglong Liu<sup>I</sup>, Fei Zhang<sup>II</sup>, Haihua Liu<sup>III</sup>, Fang Wei<sup>IV</sup>

<sup>I</sup>MD, Associate chief Physician, Department of Anaesthesiology, Gaoyou People's Hospital, Gaoyou Hospital Affiliated, Soochow University, China. Conception and design of the study, manuscript preparation, final approval.

<sup>II</sup>MD, Physician, Department of Anaesthesiology, Gaoyou People's Hospital, Gaoyou Hospital Affiliated, Soochow University, China. Acquisition, analysis and interpretation of data; manuscript preparation.

<sup>III</sup>MD, Physician, Department of Neurology, Gaoyou People's Hospital, Gaoyou Hospital Affiliated, Soochow University, China. Technical procedures, acquisition of data.

<sup>IV</sup>MD, Physician, Department of Radiology, Gaoyou People's Hospital, Gaoyou Hospital Affiliated, Soochow University, China. Analysis and interpretation of data, critical revision.

## Abstract

**Purpose:** To evaluate the role of CX3CL1 and NF- $\kappa$ B in the lumbar disc herniation induced neuropathic pain.

**Methods:** After LDH induced by implantation of autologous nucleus pulposus (NP) on the left L5 nerve root was established, mechanical thresholds and thermal hyperalgesia were tested at relevant time points during an observation period of 28 days. Expression of CX3CL1 and NF- $\kappa$ B in the dorsal root ganglion (DRG) were performed by using Western blotting and RT-PCR.

**Results:** Implantation of autologous nucleus pulposus (NP) induced neuropathic pain, associated with increased mRNA and protein expression of CX3CL1 in the DRG. Moreover, intrathecal injection of neutralizing antibody against CX3CL1 could attenuates LDH-induced persistent pain hypersensitivity. Interestingly, NF- $\kappa$ B activation in the DRGs were found in LDH-induced neuropathic pain. Furthermore, NF- $\kappa$ B downregulation by p65 inhibitor PDTC markedly alleviated LDH-induced mechanical allodynia and thermal hyperalgesia in rat. Importantly, CX3CL1 neutralizing antibody (10  $\mu$ g/10  $\mu$ l, i.t.) reduces p-p65 protein level in DRG

**Conclusions:** CX3CL1 could regulate LDH-induced neuropathic pain through NF- $\kappa$ B pathway. Targeting CX3CL1 and NF- $\kappa$ B may represent a potential treatment for neuropathic pain caused by LDH.

**Key words:** Neuralgia. Chemokine CX3CL1. NF-kappa B. Ganglia, Spinal. Rats.

## ■ Introduction

Nowadays, Long-term chronic pain compromises the patients' life quality seriously. The disc herniation associated sciatica, which is the most common types of neuropathic pain, is a common cause of low back pain and sciatica. It is mainly caused by both mechanical lumbar nerve root deformation and local inflammation induced resulting from nucleus pulposus (NP)<sup>1-3</sup>. Although previous studies suggested that various factors, including hereditary and age, might contribute to LDH-induced neuropathic pain; the pathophysiology cause for LDH-induced pain is not well understood. Moreover, it should be noted that current therapies could hardly minimize neuropathic pain. Thus, it is of great importance to dissect the mechanism of LDH-induced neuropathic pain.

Chemokines belong to a family of proinflammatory cytokines, which contribute to cell growth, development, immune system regulation and inflammation<sup>4</sup>. In experimental studies, autologous nucleus pulposus (NP) implantation in the dorsal root ganglion (DRG) is used to initiate LDH in rats. Recent studies have reported that several chemokines were involved in the NP-induced neuropathic pain<sup>5-6</sup>. For example, it has been reported that IL-8 mRNA expression was increased in the DRG after NP exposure while IL-8 inhibition could attenuated NP-induced allodynia in the spinal cord<sup>6</sup>. Moreover, CCL2/CCR2 expression were also persistently increased in the dorsal root ganglion and spinal cord after NP implantation in rats<sup>5</sup>.

CX3CL1, also named as fractalkine, plays an important role in the regulation of chronic pain by binding with its preferred receptor CX3CR1. For example, it has been found that CX3CL1 expression was increased in spinal neurons and astrocytes after NP exposure<sup>7</sup>. Enhanced expression of CX3CL1 at mRNA and protein levels were reported both in the DRG and spinal cord (SC) in multiple

sclerosis-induced neuropathic pain model<sup>8</sup>. However, the expression of CX3CL1 in the DRG in LDH rats are not studied; whether modification of CX3CL1 could attenuate LDH-induced neuropathic pain remains uninvestigated.

Nuclear factor-kappa B (NF- $\kappa$ B) is a transcription factor that has a pivotal function in the process of inflammation. It is well known that activated NF- $\kappa$ B could affect pain behavior via regulating inflammatory cytokines. Recent studies demonstrate that NF- $\kappa$ B decoys could suppresses cytokine expression in DRG and reduce hyperalgesia in both peripheral inflammatory pain model<sup>9</sup> and rat LDH model<sup>[10]</sup>. However, whether NF- $\kappa$ B pathway is involved in CX3CL1 regulated LDH-induced neuropathic pain is still unclear. In our study, we found that CX3CL1 in the DRG participated in LDH-induced neuropathic pain via NF- $\kappa$ B pathway.

## ■ Methods

The Institutional Animal Care and Use Committee of Soochow University specifically approved this study.

Experiments were performed on adult male Sprague Dawley rats weighing 200-220g. Animals were housed in separated cages with free access to food and water at 24°C temperature and under a 12/12 hour light/dark cycle. All experimental and surgical procedures were carried out in accordance with the guidelines of the International Association for the Study of Pain<sup>11</sup>. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### *Disc herniation model*

Surgeries for the disc herniation model were performed as described in previous studies<sup>10,12,13</sup>. In brief, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally) and placed in a

prone position. Laminectomy was performed to expose the left L5 nerve roots and DRG. The autologous NP was harvested from the C2-C3 intervertebral discs and was implanted next to the left L5 nerve root just proximal to the DRG in the NP group. The rats in the sham group underwent the same surgical procedure except for implantation of NP. After surgery, the rats were housed in individual cages in the animal room until they fully recovered.

### *Behavior test*

Rats were evaluated for mechanical allodynia and thermal hyperalgesia. The Behavior test was performed prior to surgery (day 0) and on days 1, 3, 5, 7, 10, 14, 21 and 28 after surgery. As described in previous study, mechanical thresholds were evaluated with the von Frey filaments (0.69–28.84g force; Stoelting, Wood Dale, IL) to calculate the 50% probability thresholds for mechanical paw withdrawal<sup>12</sup>. The rats left plantar surface of the hind paw, corresponding to the surgery, was stimulated in the L5 spinal nerve innervation area by the filaments, beginning with the 0.69g filament. The filament was applied to the paw surface for about 3s in a stepwise ascending or descending order following negative or positive withdrawal responses until six consecutive responses were noted.

Thermal hyperalgesia was measured by foot withdrawal latency to heat stimulation according to the previous studies<sup>6,13</sup>. Briefly, each rat was placed in a Plexiglas box containing a smooth glass floor, the temperature maintaining at 28°C. The heat source was provided by the analgesia meter (IITC Model 336 Analgesia Meter, Life Science) when measuring the latency to withdrawal from a thermal stimulus. The heat stimulus turned off manually when the hind paw moved (the maximum of 20 s to prevent tissue damage). Each rat was taken 5 tests of each hind paw, 30 s apart, at 10 mins intervals. A total mean data at each time point was

calculated for each hindpaw<sup>14</sup>.

### *Real-time PCR*

Total RNA was isolated from the left L5 DRG tissues at 0, 1, 5, 10, and 21 days after surgery. The protocol for real-time PCR was described as previous papers<sup>8</sup>. A SYBR green I kit was used to perform the PCR reaction following manufacturers protocols (Bio-Rad, Hercules, CA, USA). To amplify rat CX3CL1, the following primers were used: CX3CL1 sense primer 5'-GAA TTC CTG GCG GGT CAG CAC CTC GGC ATA -3' and antisense primer 5' -AAG CTT TTA CAG GGC AGC GGT CTG GTG GT -3'; GAPDH sense primer 5'-ACC AGG GCT GCT TTT AAC TCT G-3' and antisense primer 5'- CCT TGA CTG TGC CGT GGA AC -3'. PCR was performed by 40 amplification cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 60 seconds. Expression levels were normalized to GAPDH.

### *Western blot*

Western blot analysis was performed according to the manufacturer's protocol as previous reported<sup>15</sup>. L5 DRGs were immediately removed from deeply anesthetized rats and homogenized on ice in the lysis buffer (Cell Signaling, Danvers, MA). Equal amount of samples was run on a 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After 1h blocking in the block buffer (5% nonfat dried milk), the membranes were incubated with the primary antibodies against CX3CL1 (1:500, R&D Systems, USA), NF- $\kappa$ B p65 (1:1000, Abcam, USA), phosphorylated NF- $\kappa$ B p65 (Ser311; 1:1000, CST, USA), and GAPDH (1:20000, Sigma, USA) at 4°C overnight. The membranes were then incubated with a horseradish peroxidase-labeled secondary antibody. ECL detection kit (Millipore, Billerica, MA, USA) were used to detect the immune complex. Protein expression was normalized against GAPDH. Western blot

bands were quantified with the Quantity One image software (Bio-Rad).

### Statistical analysis

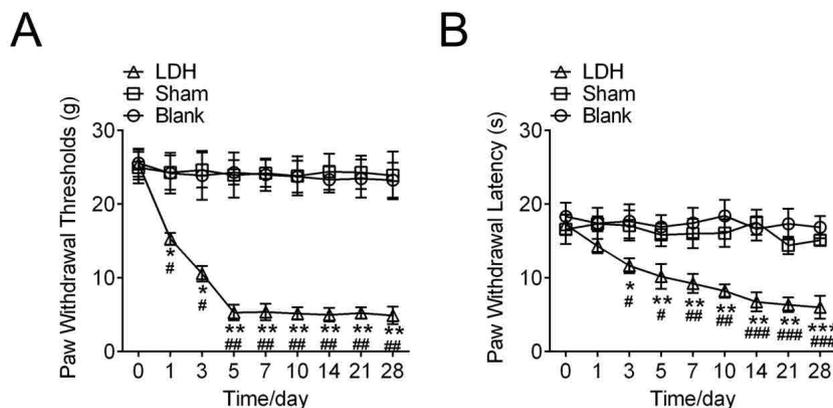
All data were expressed as the means $\pm$ SEM and analyzed using the Tukey *post hoc* test by SPSS 13.0 (SPSS, USA). *P* values of less than 0.05 were considered significant.

## Results

### Behavioral test

All rats shown stable responses to mechanical stimulation before surgery. In

the rats with LDH, mechanical withdrawal threshold showed a significant decrease 1 day after the surgery to 28 days after the surgery, compared with the Blank group (rats without the surgery) and the Sham group (Rats underwent the surgery without implantation of NP). There is not any significant difference in the mechanical withdrawal threshold between Blank group and Sham group (Figure 1A). Moreover, similar results were shown on the thermal withdrawal latency study. Compared to the Sham group, thermal withdrawal latency reduced markedly in the rats with LDH from 1 day after the surgery and did not recover during the behavioral test (Figure 1B).

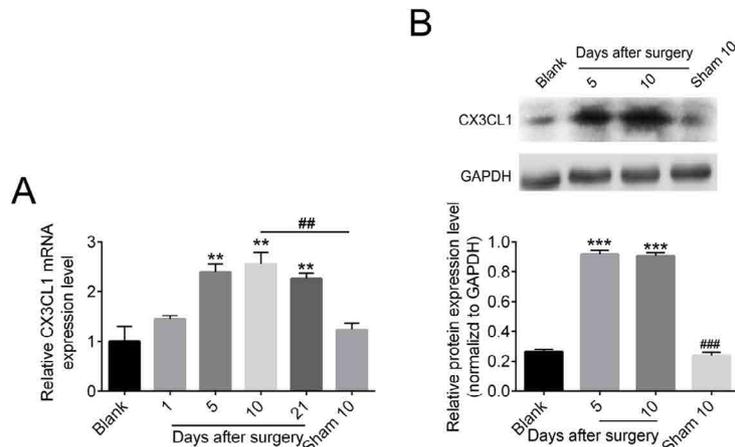


**Figure 1** - Behavioral responses to mechanical stimulation of hind paw and heat in lumbar disc herniated models. Animals in lumbar disc herniated groups experienced significant mechanical allodynia (A) and thermal hyperalgesia (B) that started at day 1 and continued through day 28, compared with the Blank group and Sham group. Data are the means  $\pm$  SEM (n=10 in each group, \**P*<0.05, \*\**P*<0.01, compared with Blank group, #*P*<0.05, ##*P*<0.01, compared with Sham group ).

### Expression of CX3CL1 to the LDH rats in the DRG

We then measured CX3CL1 mRNA expression in the DRG at 0, 1, 5, 10, 21 days after surgery by RT-PCR. The statistical results showed that the CX3CL1 mRNA level was significantly increased at 5 days after surgery (*P*

< 0.05) and peaked at 10 days after surgery (*P*< 0.01) (Figure 2A). CX3CL1 mRNA level at 10 days after surgery was also significantly higher than that in the sham group (*P*< 0.01; Figure 2A). Furthermore, western blot data demonstrated that CX3CL1 expression in the 5 and 10 days after surgery increased greatly comparing with Blank group (*P*< 0.05; Figure 2B).

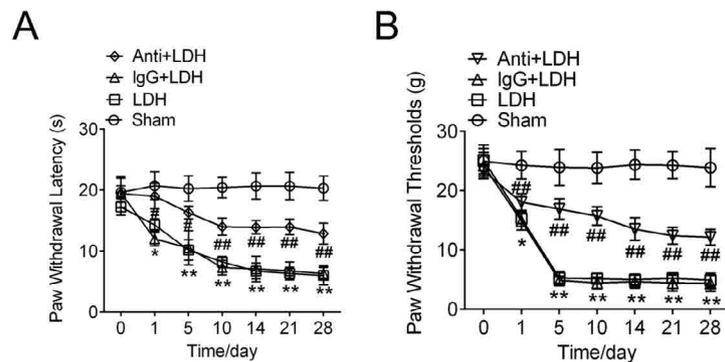


**Figure 2** - Expression of CX3CL1 in the DRG of LDH rats. DRG of LDH rats and control groups were collected for analysis in the presented time points. Representative histogram and blots shown the upregulation of CX3CL1 in the DRG of LDH rats, both in (A) mRNA level and (B) protein level. Data are the means ± SEM (n=5 in each group, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, compared with Blank group, #P<0.05, ##P<0.01, ###P<0.001, compared with LDH 10 day group ).

*CX3CL1 downregulation attenuates LDH-Induced neuropathic pain*

As both the RNA and protein expression of CX3CL1 was increased in DRG after LDH surgery, we speculated that CX3CL1 may contribute to the LDH-induced pain behaviors. Rats were consecutively intrathecal injected neutralizing antibody against CX3CL1 (10 μg/10 μl, R&D system) or IgG antibody (10

μg/10 μl, R&D system) as negative control for 10 days after LDH surgery and then the PWT and PWL were tested. The data in PWT and PWL test were found similar which showed that compared to the LDH group; continuous intrathecal injection of neutralizing antibody against CX3CL1 could attenuates thermal hyperalgesia induced by LDH model, whereas the IgG injection could not change paw withdrawal latency (Figure 3 A, B).



**Figure 3** - Pre-treatment with CX3CL1 neutralizing antibody (10 μg/10 μl, i.t.) but not IgG (10 μg/10 μl, i.t.) suppresses LDH-induced mechanical allodynia (A) and thermal hyperalgesia (B) Data are the means ± SEM (n=10 in each group, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared with Sham group, #P<0.05, ##P<0.01, ###P<0.001 compared with LDH group).

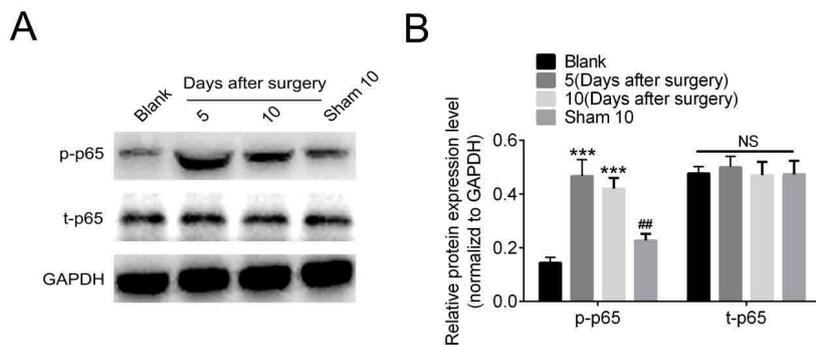
*NF-κB activation in the DRGs of LDH rats*

It is well-known that NF-κB contributes to neuropathic pain<sup>16</sup>. Here, we investigate

whether NF-κB signaling is activated in our LDH model rats. We measured the expression of both phosphorylated and total NF-κB p65 in DRG at 5 and 10 days after surgery.

The data demonstrated that the expression phosphorylated p65 (p-p65) at 5 and 10 days after the surgery was significantly increased in DRG compared with the Blank group without

surgery. P-p65 value was not between the Blank group and the Sham group 10 days after the surgery. Moreover, the protein expression of total p65 were similar in each group (Figure 4).

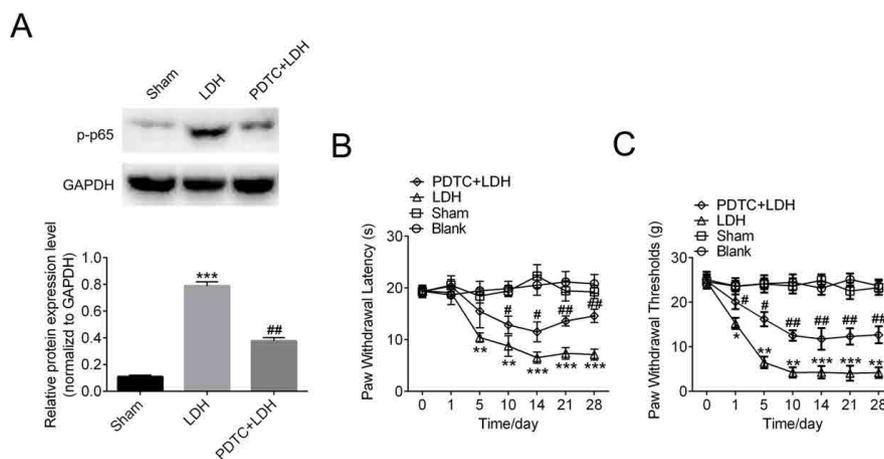


**Figure 4** - NF- $\kappa$ B activation in the DRGs of LDH rats. DRG of LDH rats and control groups were collected for analysis in the presented time points. Representative histogram and blots shown the expression level of p-p65 and t-p65. Data are the means  $\pm$  SEM (n=5 in each group, \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, compared with Blank group, # $P$ <0.05, ## $P$ <0.01, compared with LDH 10 day group ).

#### Downregulation of NF- $\kappa$ B attenuates LDH-Induced neuropathic pain

Next, NF- $\kappa$ B p65 inhibitor PDTC (200 ng/10 ml for 10 days) were continuously intrathecal injected to the rats after the LDH surgery to downregulate the NF- $\kappa$ B activation. After the injection of PDTC, the expression of

p-p65 was downregulated compared to the LDH group, which indicated the efficiency of p65 inhibitor PDTC (Figure 5A). Importantly, the data from behavior test PWT and PWL showed that continuous intrathecal injection of PDTC could attenuates thermal hyperalgesia induced by LDH model, compared to the LDH group (Figure 5 B, C).

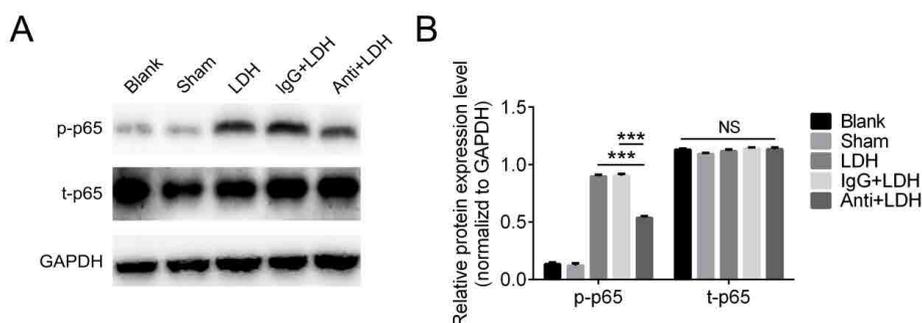


**Figure 5** - Downregulation of NF- $\kappa$ B attenuates LDH-Induced neuropathic pain. LDH rats were injected with p65 inhibitor PDTC (200 ng/10 ml for 10 days), DRG of LDH rats and control groups were collected for analysis. (A) Representative histogram and blots shown the decreased activation of p65. Data are the means  $\pm$  SEM (n=5 in each group, \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, compared with Sham group, # $P$ <0.05, ## $P$ <0.01, compared with LDH group ). (B, C) Intrathecal injection of PDTC obviously attenuated the mechanical allodynia and thermal hyperalgesia caused by LDH (n=10 in each group, \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 compared with Sham group, # $P$ <0.05, ## $P$ <0.01, ### $P$ <0.001 compared with LDH group ).

### CX3XL1 regulates LDH-induced neuropathic pain through NF- $\kappa$ B pathway

Knowing the fact that NF- $\kappa$ B pathway plays a cardinal role in regulating LDH-induced neuropathic pain; we want to specifically study the function of CX3XL1 in the signaling pathway. We neutralized the CX3XL1 by injection antibody against it or IgG as control to the rats. It has been found that total p65 level were not

affected too much, either in antibody group or IgG group when compared with blank or sham control. Consistently, the p-p65 level increased dramatically in LDH group. Interestingly, p-p65 was significantly inhibited in LDH rats injected with anti- CX3XL1 antibody; while the one with IgG antibody shown similar p-p65 level with LDH group (Figure 6). These data strongly suggests that CX3XL1 regulates LDH-induced neuropathic pain by activating NF- $\kappa$ B pathway.



**Figure 6** - CX3XL1 regulates LDH-induced neuropathic pain through NF- $\kappa$ B pathway. CX3CL1 neutralizing antibody (10  $\mu$ g/10  $\mu$ l, i.t.) but not IgG (10  $\mu$ g/10  $\mu$ l, i.t.) reduces p-p65 level, while the total p65 level are not affected in each group. Representative histogram and blots shown the expression level of p-p65 and p65 after antibody injection. Data are the means  $\pm$  SEM (n=5 in each group \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, compared with Anti+LDH group).

## Discussion

Nowadays, LDH is accounting for 80% of patients that suffering from back pain<sup>17</sup>. However, the molecular mechanism underlying this disease is still not clear. A series of studies has used implantation of autologous NP in rats to mimic LDH pain, which were also adopted by us<sup>6,18</sup>. In this paper, we firstly reported the involvement of CX3XL1 in LDH-induced neuropathic pain. As its upregulation were identified in the DRG of LDH rats both by RT-PCR and western blot. Moreover, inhibition of CX3XL1 activity by specific antibody injection significantly attenuated LDH-Induced neuropathic pain. Furthermore, we have found that NF- $\kappa$ B pathway was activated in this event. And using NF- $\kappa$ B p65 inhibitor PDTC

also rescued the neuropathic pain induced by LDH. More importantly, our data suggests that CX3XL1 was responsible for the activation of NF- $\kappa$ B pathway in LDH rat; which provides a new insight in LDH-induced neuropathic pain study.

It has been widely reported that neuroinflammation is one of the leading cause for neuropathic pain<sup>19</sup>. Chemokines, which belongs to the family of small cytokines, is the main mediator for the response of inflammation. They are divided into four subgroups according to their structures. CX3XL1, which characterized by its CX3C motif, is the only member of fourth chemokine group. There are two different forms of CX3XL1 exists in cells: one is membrane bounded and the other one is soluble form after cleavage in

cytosol<sup>20</sup>. By binding with its receptor CX3CR1, CX3CL1 is mainly found to be expressed in different types of neuronal cells or NK cells, T cells and smooth muscle cells<sup>21</sup>. Recent studies indicate that CX3CL1 were found in some tumor cells, such as breast cancer cell and ovarian cancer cells<sup>22,23</sup>. However, the focus of CX3CL1 study is still on varieties of neurological disorders, such as peripheral neuropathy, experimental autoimmune encephalomyelitis and rheumatoid arthritis<sup>24</sup>. Our study is the first to report the involvement of CX3CL1 in LDH induced neuropathic pain.

NF- $\kappa$ B is a cardinal transcriptional factor that involved in the response of inflammation and cancers<sup>25</sup>. Moreover, more studies have been done on NF- $\kappa$ B related to chronic pain. For instance, recent studies have shown its critical role in chemotherapy-induced chronic pain<sup>26</sup>. We were specifically interested the molecular mechanism in LDH induced pain. Interestingly, NF- $\kappa$ B was activated in LDH rats and its activation was regulated by the expression of CX3CL1. For further studies, as it is well known that Akt, which is activated by phosphatidylinositol 3-kinase (PI3K), could phosphorylates NF- $\kappa$ B's inhibitor  $\kappa$ B (I $\kappa$ B). This phosphorylation could leads to ubiquitination and degradation of I $\kappa$ B; thus leading to the activation of NF- $\kappa$ B<sup>27</sup>. However, how this signaling pathway is regulated in LDH rats is still unclear, our further study will focus on PI3K-Akt signaling.

## ■ Conclusions

CX3CL1 could regulate LDH-induced neuropathic pain through NF- $\kappa$ B pathway. Targeting CX3CL1 and NF- $\kappa$ B may represent a potential treatment for neuropathic pain caused by LDH.

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**Correspondence:**

Chenglong Liu  
Department of Anaesthesiology  
Gaoyou People's Hospital, Gaoyou Hospital  
Affiliated  
Soochow University  
Yangzhou, Jiangsu 225600 China

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