

# Down-Regulation of NOX4 Expression in Dorsal Horn of Spinal Cord Could Alleviate Cancer-Induced Bone Pain in Rats by Reducing Oxidative Stress Response

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**Introduction:** Cancer-induced bone pain (CIBP) is very common in patients with advanced cancer. Recent studies have shown that reactive oxygen species (ROS) can sense and regulate pain response process through spinal cord mechanism, and play a role in CIBP. NADPH oxidase (NOX) is a group of protease complexes that produce ROS. In the current study, we investigated the expression of NOX4 in the spinal dorsal horn of rats with CIBP and its related role and molecular mechanism.

**Materials and Methods:** A rat CIBP model was established by injecting Walker-256 cells into the tibia medullary cavity, and the expression of NOX4 in spinal dorsal horn was down-regulated by injecting lentivirus into spinal cord. RT-PCR, Western blot and immunofluorescence staining were used to detect the expression of NOX4 in CIBP rats, cell localization and its effect on CIBP rats, and the effect of down-regulating the expression of NOX4 on the expression of H<sub>2</sub>O<sub>2</sub>, nitric oxide synthase nNO, antioxidant enzyme SOD, and the activity of neuro-receptor in spinal dorsal horn of rats.

**Results:** In rats with CIBP, the expression of NOX4 was significantly increased, and immunofluorescence showed that NOX4 was mainly expressed in microglia in the dorsal horn of spinal cord. Down-regulating the expression of NOX4 in rats can reduce the level of H<sub>2</sub>O<sub>2</sub> and nNO in dorsal horn tissue, and increase the expression of SOD to reduce the oxidative stress response. At the same time, down-regulating NOX4 can reduce the sensitivity of spinal cord and relieve the pain of bone cancer by inhibiting the expression of NMDAR and GABAA- $\gamma$ 2 in dorsal horn tissue.

**Conclusion:** NOX4 is a promising therapeutic target in CIBP, and down-regulation of NOX4 expression can alleviate CIBP in rats by reducing oxidative stress and weakening spinal cord sensitization.

**Keywords:** cancer-induced bone pain, NOX4, reactive oxygen species, spinal cord sensitization

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## Background

Cancer is one of the major causes of death worldwide. Although novel therapeutic strategies have significantly improved the survival of most patients, the pain caused by cancer seriously affects their life quality.<sup>1,2</sup> Cancer-induced bone pain (CIBP) refers patients with primary bone tumors (sarcomas and hematopoietic malignancies) and more commonly in patients with bone cancers that have metastasized from

distant sites such as breast, prostate, or lung.<sup>2</sup> The pathogenesis of CIBP is complex and has not yet been fully elucidated. Studies show that sustained stimulation of peripheral nociceptors by secreted tumorigenic, osteolytic and inflammatory factors, as well as periosteal traction or compression of peripheral nerves, blood vessels and other tissues due to tumor invasion sensitizes the peripheral neurons to pain. In addition, central sensitization due to aberrant release of excitatory and inhibitory neurotransmitters and overactivation of corresponding receptors is also closely related to CIBP, although the specific mechanism is still unclear.<sup>3-5</sup>

Nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase (NOX) is a group of protease complexes that produce reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ), superoxide anion, nitric oxide, etc., during oxidative phosphorylation.<sup>6,7</sup> The NOX family of proteases are the major catalysts of ROS generation and oxidative stress. NOX4 was the first kidney-specific NOX to be discovered, and has since been detected in nerve cells and vascular smooth muscle cells, and in organelles including mitochondria, nuclei and endoplasmic reticulum. And NOX4 is distinct from other NOX isomers on account of its tissue-specific expression and unique molecular structure. In addition, while activation of NOX1-3 depends on the phosphorylation of cellular subunits and that of NOX5 on  $Ca^{2+}$  signaling, NOX4 harbors an intrinsic-activated dehydrogenase domain that can generate ROS through the electron transfer from NADPH to flavin adenine dinucleotide (FAD). However, due to the highly conserved histidine residue in its third extracellular ring, NOX4 could induce superoxide to spontaneously disproportionate into  $H_2O_2$  in the enzyme, and therefore the ROS produced by NOX4 is mainly  $H_2O_2$ .<sup>8,9</sup> Under normal circumstances, the baseline amounts of ROS are neutralized by superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase ( $GP_x$ ) and other antioxidants, which maintain a dynamic balance between ROS generation and elimination.<sup>6,7</sup> However, chronic and stressful stimuli can trigger a surge in ROS levels that overwhelm the antioxidant system, leading to oxidative stress and a pathological state. Recent studies show that ROS is involved in regulating sensory transmission and its high levels are responsible for nerve injury-related chronic pain. For instance, ligation of the L5 spinal nerves in a rat model of neuropathic pain significantly increased ROS production in the microglia of dorsal horn.<sup>10</sup> In addition, Zhou et al<sup>11</sup> recently showed that the ROS scavenger

N-tert-Butyl- $\alpha$ -phenylnitron (PBN) and tetramethylpiperidine (4-hydroxy-2, 2,6,6-tetramethylpiperidine-1-oxyl, Tempol) reduced the mechanical pain in a rodent CIBP model by inhibiting dorsal horn microglial activation. These findings indicate that ROS is likely involved in the perception and regulation of pain through the spinal cord, and therefore a causative factor of CIBP. Accordingly, we hypothesized that down-regulating NOX4 in the dorsal horn tissues would alleviate pain by inhibiting ROS production. To this end, we established a rat model of CIBP and specifically knocked down NOX4 in the dorsal horn neurons through intrathecal lentiviral injection. The changes in pain threshold, ROS production and other related indicators were measured in order to determine the role of NOX4 in CIBP and the underlying mechanisms.

## Materials and Methods

### Cell Lines and Main Reagents

Waiker256 rat breast cancer cells were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco. RIPA lysate and BCA protein quantification kit were purchased from Invitrogen, and the reverse transcription and real-time quantitative PCR kit from TaKaRa. Lentiviral vectors containing enhanced green fluorescent protein (EGFP) were employed in experiments to stably knockdown NOX4. The vectors GV369 (Ubi-MCS-SV40-EGFP-IRES-puromycin) and NOX4 (NM\_001143836) were recombinant (Shanghai KeyGen Biotech Co. LTD, Shanghai, China). The same vector framework, without genes but carrying EGFP, was used as a negative lentivirus control (NC-LV). The 293T cells were transfected with lentiviral vectors at a density of  $5 \times 10^6$  cells/15 mL. After amplification and concentration, the viral titer of the RNAi-LV was  $2.0 \times 10^8$  TU/mL. Rabbit anti-NOX4 and anti-GAPDH monoclonal antibodies were procured from Abcam, mouse anti-SOD and nNOS from Santa Cruz, goat anti-NMGAR2D and GABAA- $\gamma 2$  from CST company, and the rabbit anti-NOX4, goat anti-NeuN, anti-Iba1 and anti-GAFP antibodies from Millipore. Alexa Fluor 488 and Alexa Fluor 555 labeled donkey anti-goat/rabbit IgG were purchased from Guangzhou Jingcai Biological, and the HRP-labeled goat anti-rabbit/mouse IgG, immunocytochemical staining kit and HE staining kit from Wuhan Boster Biological Technology Co. Ltd. The ROS Detection Kit was purchased from Nanjing Jiancheng

Biological Engineering. All PCR primers were synthesized by Shanghai Biotech.

## Intrathecal Catheterization and Lentiviral Injection

SPF-grade healthy female SD rats weighing (180~220) g were purchased from Vital River Company and housed at 22–24°C, 55% humidity and a 12 h diurnal cycle, with ad libitum access to food and water. The rats were divided randomly into the sham operated (Sham), untreated CIBP (CIBP), empty vector (CIBP+NC-LV) and NOX4 knock-down (CIBP +RNAi-LV) groups (n=16 in each group) and treated accordingly. All animal experiments were approved by the ethics committee of Xinjiang Medical University (XJMU-20190017, Xinjiang, China), and conducted in strict accordance with the guidelines of the International Association for Study of pain on the use of animals for pain experimental research. The rats were anesthetized with isoflurane (3% for induction and 2% for maintenance). Subarachnoid catheterization was performed in the lumbosacral region as reported previously.<sup>12,13</sup> Briefly, a PE-10 catheter was inserted into the subarachnoid space through the cerebello-medullary cistern to a depth of 7–7.5 cm along the direction of the spinal cord towards the intumescencia lumbalis of the tail. A 3–0 silk thread was then passed through the gap of the catheter to suture the muscle and fix the catheter in position. After excluding rats with nerve dysfunction or catheter detachment 20 h after catheterization, the guide tube was injected into the remaining animals with 10 mL 2% lidocaine. Paralysis in the hind limbs confirmed correct positioning of the catheter, following which the rats were injected intraperitoneally with 5 mL normal saline. On the third day after intrathecal catheterization, 10  $\mu$ L RNAi-LV or NC-LV was injected daily through the intrathecal catheter for 3 consecutive days. The sham-operated animals were injected with an equal volume of normal saline. All rats of the same groups were housed in a single cage after catheterization.

## Establishment of CIBP Model

Walker 256 cells were maintained in RPMI-1640 medium containing 10% FBS at 37°C under 5% CO<sub>2</sub>. Viable cells were harvested, re-suspended in PBS at the density of 2 $\times$ 10<sup>7</sup> cells/mL, and inoculated into the abdominal cavity of rats at the volume of 500  $\mu$ L. After one week of conventional feeding, 10 mL tumor ascites was extracted

from each animal and centrifuged at 1000 rpm for 8 min. The pelleted cells were resuspended in PBS at the density of 2 $\times$ 10<sup>7</sup>/mL, and a small aliquot was boiled for 20 min for the Sham group rats. The CIBP model was established as previously described.<sup>12</sup> Briefly, the rats were anesthetized (see section 1.2.1) on the third day after lentiviral injection and placed in supine position. The skin of the left hind limb was depilated and disinfected with iodine, and cut longitudinally at the joint of the left knee. The surface of tibial plateau was fully exposed by blunt dissection of the muscle, and the bone was drilled perpendicular to the surface with a No.5 needle till the bone marrow cavity. The needle was then pulled out, and 10  $\mu$ L (~2 $\times$ 10<sup>5</sup> cells) of the live or dead Walker256 suspension was injected slowly into the cavity using a 25  $\mu$ L microsyringe. The latter was drawn after 30 s, and the injection hole was closed with sterile bone wax. After sterilizing the wound, the muscle and skin layers were sutured.

## Analgesic Behavioral Testing

The calibrated Von-Frey cilia were used to determine the paw withdraw threshold (PWT) of the hind foot on the affected side. As previously reported by Tong et al<sup>14</sup> the rats were acclimatized for 30 min prior to the experiment and the bottom of the third and fourth feet of the right hind foot were stimulated by the tip of Von-Frey cilia. The stimulation intensity was steadily increased from 0 to 40 g within 20 s. The time taken for the rat to withdraw or move the foot was recorded as the PWT. Paw withdrawal latency (PWL) of the middle and posterior 1/3rd of the right plantar was evaluated in response to thermal pain. The intensity of thermal irradiation was adjusted to 10 V and 50 W, and the diameter of the stimulated spot was 0.8 cm.<sup>15,16</sup> The reaction time of retracting, adding or lifting the foot, or biting was recorded as PWL. All measurements were taken between 8:00 a.m. to 12:00 noon by the same technicians, and 16 rats in each group were measured in batches, and each item was measured three times at 3 min intervals. The PWT and PWL scores were evaluated before and on days 0, 3, 7, 14 and 21 post-intrathecal catheterization.

## Histopathology

The rats were deeply anesthetized by intraperitoneal injection of 10% chloral hydrate on the 21st day after CIBP induction and dissected. Eight rats in each group were randomly selected for HE staining. The affected tibia was removed and decalcified in 15% EDTA-2Na for 3 weeks, with the solution replaced weekly. After washing

with PBS, the decalcified tibia was embedded in paraffin and sectioned. The tissue sections were dewaxed, dehydrated through an alcohol gradient, and stained by HE before microscopic examination.

## Spinal Dorsal Horn Harvest and Immunohistochemistry (IHC)

The rats were anesthetized as above, and the dorsal region 2 cm on either side of the median line as well as both hind limbs was depilated. The animals were dissected along the median dorsal line from the tail to the upper part of the hind limbs, and the subcutaneous tissues and muscles were separated to expose the spine. The L4-6 section of the spinal cord harboring the spinal dorsal horn tissue was extracted from 8 rats randomly in each group, washed with chilled PBS and frozen in liquid nitrogen for molecular assays. The remaining 8 rats in each group underwent IHC staining of spinal dorsal horn tissue, the chest wall of the anesthetized rats was opened to expose the heart, and 10 mL normal saline was injected into the aorta through the left ventricle following exsanguination. After the saline wash, the animals were perfused with 4% polyformaldehyde, and the dorsal horn tissues were removed and fixed in 4% paraformaldehyde for 2 h. The fixed tissues were rinsed with PBS, dehydrated in 30% sucrose solution, embedded in optimal cutting temperature compound (OCT), and frozen at  $-20^{\circ}\text{C}$  for cryosectioning. The sections were rinsed thrice with PBS, immersed in blocking solution for 1 h at room temperature, and incubated overnight with anti-NOX4 (1:500) and anti-NeuN (1:200), anti-GFAP (1:300) or anti-Iba 1 (1:300) antibodies at  $4^{\circ}\text{C}$ . After washing thrice with PBS, the sections were incubated with Alexa Fluor 488 and Alexa Fluor 555 labeled secondary antibodies for 2 h at room temperature. Following a final wash cycle, the stained sections were observed and imaged under a confocal microscope.

## Detection of in situ $\text{H}_2\text{O}_2$

The dorsal horn tissues were washed thrice with chilled PBS and homogenized in RIPA lysis buffer. The lysates were centrifuged at 12,000 rpm at  $4^{\circ}\text{C}$  for 5 min and the supernatants were aspirated. The concentration of  $\text{H}_2\text{O}_2$  was measured using the specific kit as per the manufacturer's instructions.

## Real-Time Fluorescence Quantitative PCR (RT-PCR)

Total RNA was extracted from the dorsal horn tissues using Trizol, and the purity and concentration were detected using a spectrophotometer. RNA was reverse transcribed into cDNA using a reverse transcription kit, and RT-PCR was performed according to the kit instructions and reaction conditions standardized by preliminary experiments. The RT-PCR primer sequences are as follows:

NOX4-F:5'-TCTGGAAAACCTTCCTGCTG-3', NOX4-R:5'-CCGGCACATAGGTAAGGA-3'; NMGAR2D-F: 5'-GCCCTGGCAGTGTCTTAG-3', NMGAR2D-R:5'-CAGT GCGTGTCTGGAG-5'; GABAA- $\gamma$ 2-F:5'GCAGCAGCGG CTTACA-3', GABAA- $\gamma$ 2-R:ACATCCAAACAGGAGCG TCAT; GAPDH-F:5' AGAAGGCTGGGGCTCATTG 3', GAPDH-5' AGGGCCATCCACAGTCTTC 3'. GAPDH was used as the internal reference, and the expression levels of the target RNA were analyzed by the  $2^{-\Delta\Delta\text{Ct}}$  method.

## Western Blotting

The dorsal horn tissues were washed thrice with chilled PBS and homogenized with RIPA lysis buffer supplemented with protease inhibitor. Following quantification with the BCA kit, 35  $\mu\text{g}$  of each protein sample was mixed with  $5\times$  loading buffer at the ratio of 1:4, denatured by boiling for 10 min, and separated by polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were transferred to PVDF membrane by wet rotation method and blocked with 5% skim milk at room temperature for 2 h. The blots were then incubated overnight with primary antibodies against SOD (1:500), nNOS (1:500), GABAA- $\gamma$ 2 (1:300), NMDAR2D (1:500) and GAPDH (1:1500) at  $4^{\circ}\text{C}$  with constant shaking. After washing thrice with TBST solution for 5 min each time, the membranes were incubated with HRP-labeled secondary antibody (1:5000) for 1 h at room temperature, and washed again as described. The positive bands were developed using an ECL reagent and exposed in a gel imager. The gray values of the bands were measured by Image J software, and the ratio of the target protein to the internal reference GAPDH was calculated. Three independent experiments were performed.

## Statistical Analysis

SPSS 19.0 and GraphPad Prism 5.0 were used for statistical analysis and all data were expressed as  $X\pm S$ . Two groups were compared using the independent sample *t*-test, and

multiple groups by one-way ANOVA and Dunnett's or Bonferroni's ad-hoc test. The significance test level was  $\alpha = 0.05$ , and  $P < 0.05$  was considered statistically significant.

## Results

### Successful Construction of CIBP Model

The induction of CIBP was verified in terms of pathological and behavioral parameters. Fourteen days after the tumor cells were inoculated into the tibia, the rats were killed under anesthesia. The tibia of the affected side of the rats was taken, and the knee joint showed swelling growth (see [Supplementary Figure 1](#)), and some rats showed obvious abnormal conditions such as licking feet, lifting feet, limping, etc. As shown in [Figure 1A](#), the tibias of the sham-operated rats were intact with a clear boundary between the bone tissue and cavity, and abundant bone marrow. In contrast, inoculation of tumor cells resulted in visible deterioration of the tissue micro-structure and replacement of the bone marrow cells with tumor cells within 7 days, which progressed to severe destruction of the bone matrix and trabeculae, obliteration of the boundary between tissue and marrow cavity, and excessive tumor cell proliferation in the bone marrow by the 14th and 21st day ([Figure 1A–D](#)). Pathological changes of the tibia at low magnification were shown in [Supplementary Figure 2](#). The basal mechanical and thermal pain thresholds were similar across all

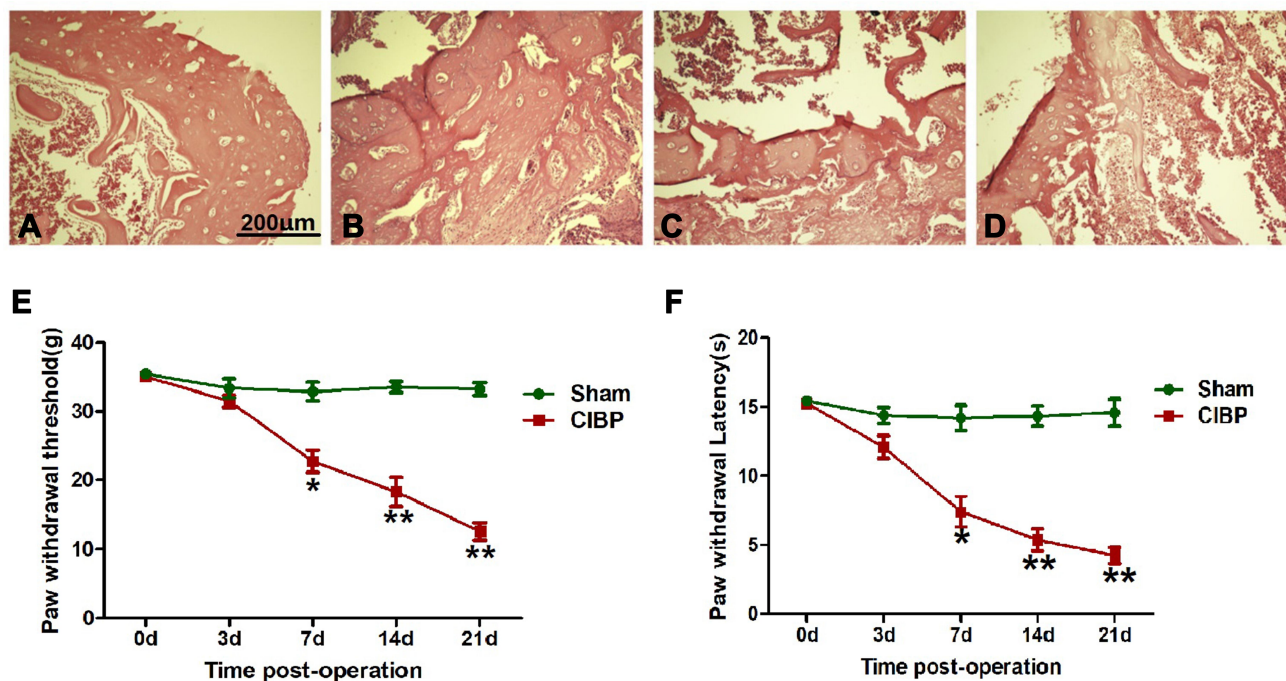
groups. However, the pain threshold of the CIBP rats decreased significantly from day 7 post-inoculation, and sensitivity to pain gradually increased over the progression of disease. In contrast, the pain threshold of the sham-operated rats was relatively stable ([Figure 1E and F](#)). Taken together, the CIBP model was successfully established.

### NOX4 is Upregulated in the Microglia Upon CIBP Induction

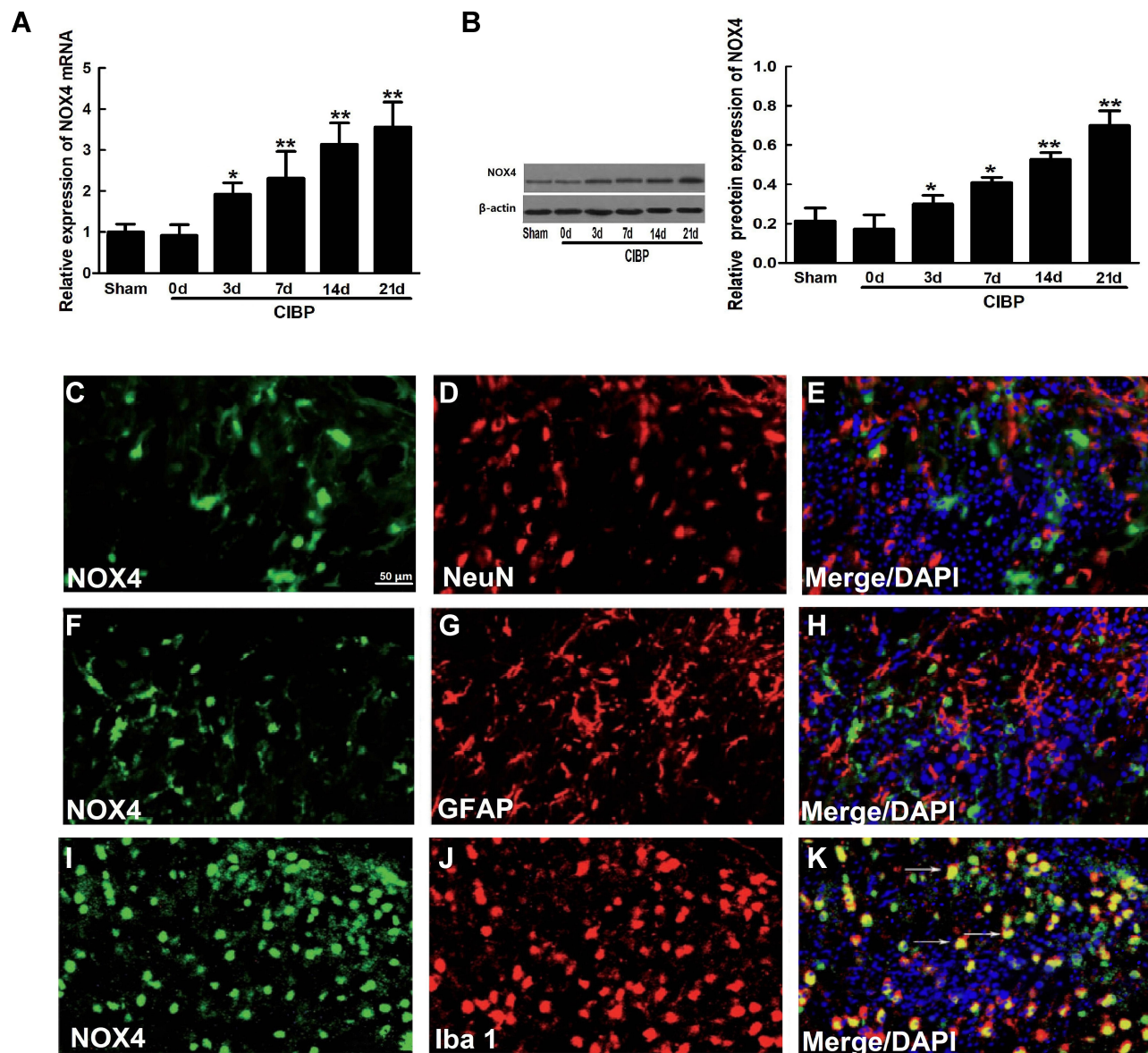
NOX4 mRNA and protein expression increased significantly in the spinal cord tissues of the CIBP modeled rats compared to the sham-operated animals and showed a time-dependent upregulation with the progression of the disease ([Figure 2A and B](#)). Furthermore, co-staining of the spinal dorsal horn tissues with neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP) or ionized calcium binding adaptor molecule-1 (Iba 1) and NOX4 from CIBP group indicated that the latter was primarily localized to the microglia as opposed to the neurons and astrocytes ([Figure 2C–K](#)). NOX4 cell localization at low magnification was shown in [Supplementary Figure 3](#).

### NOX4 Knockdown Significantly Alleviated CIBP

To further elucidate the biological relevance of NOX4 in CIBP, we knocked down the gene in the spinal tissues via



**Figure 1** Establishment of CIBP model. (A–D) Bone structure of the sham-operated and tumor-bearing rats on days 7, 14 and 21 after tumor inoculation ( $n=8$ ). (E and F) The mechanical (E) and thermal (F) withdrawal thresholds of the sham-operated and tumor-bearing rats at the indicated time points ( $n=16$ ). \* $P < 0.05$ , \*\* $P < 0.01$  compared to the Sham group.



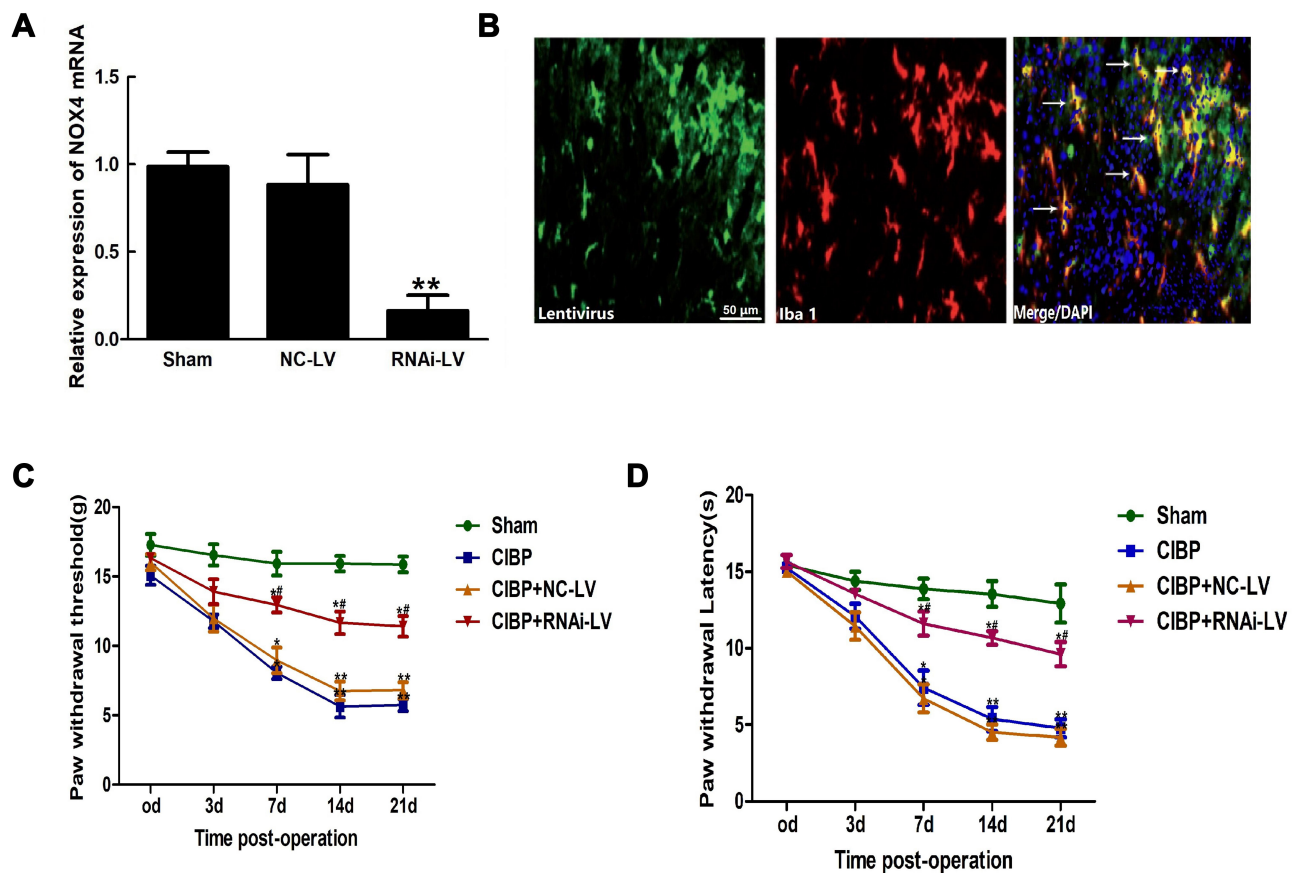
**Figure 2** The expression and cellular localization of NOX4 in spinal cord tissues. (A and B) NOX4 mRNA (A) and protein (B) levels in the spinal cord tissue of Sham and CIBP group rats (n=3). (C–K) Representative immunofluorescence images showing co-staining of NOX4 (green) with NeuN+ (red) neurons, GFAP+ (red) astrocytes or Iba1+ (red) microglia in the spinal dorsal horn of the affected side on the 21st day of CIBP group after tumor inoculation (n=8).

**Note:** \*,  $P < 0.05$  \*\*\*,  $P < 0.01$ .

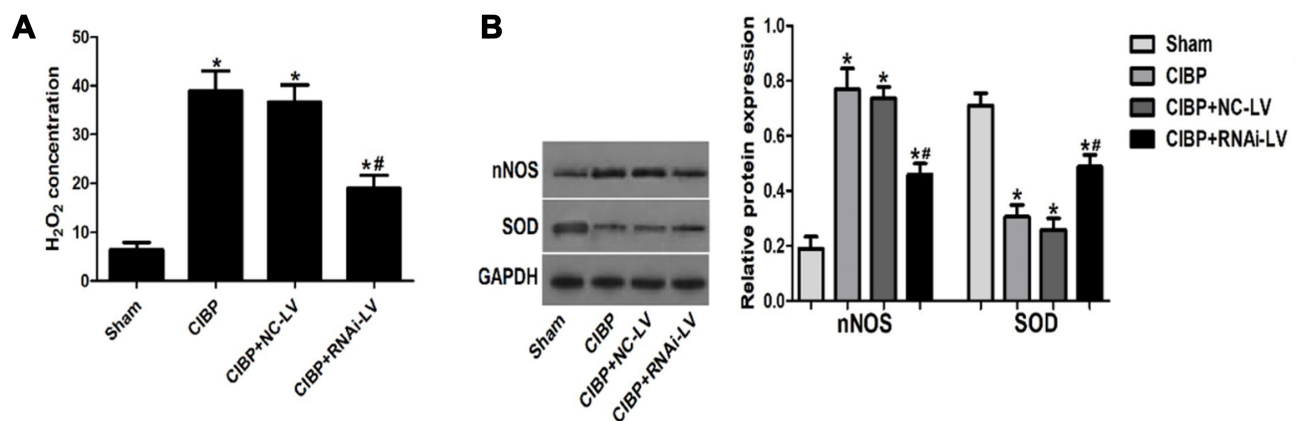
intrathecal lentiviral injection. As shown in Figure 3A, NOX4 mRNA levels were significantly downregulated in spinal tissues of the rats infected with RNAi-LV compared to the Sham and NC-LV groups. Figure 3B shows that NOX4 co-locates with microglia cells in spinal dorsal horn tissues of CIBP + RNAi-LV group, which further confirms that the lentivirus down-regulating the expression of NOX4 is successfully infected in rats. Knockdown of NOX4 significantly alleviated the mechanical and thermal pain sensitivity in the rats, thereby underscoring the causative role of NOX4 in CIBP (Figure 3C and D).

## NOX4 Knockdown Alleviated Oxidative Stress in Spinal Dorsal Horn Tissue

NOX family is the main source of reactive oxygen species (ROS), while NOX4 mainly mediates ROS in sound field as  $H_2O_2$ . In order to further explore the effect of down-regulation of NOX4 expression on  $H_2O_2$ , NO and SOD expression in dorsal horn tissues of rats, we found that down-regulation of NOX4 could significantly decrease ROS levels in dorsal horn tissue (Figure 4A), and concomitantly increase that of the antioxidant enzyme SOD (Figure 4B). ROS like the superoxide anions combine



**Figure 3** Down-regulation of NOX4 expression significantly alleviated CIBP in rats. **(A)** NOX4 mRNA levels in the spinal tissues (n=8). **(B)** Immunofluorescence images showed the co-localization of NOX4 (green) and Iba1 (red) microglia cells in the dorsal tissues of CIBP + RNAi-LV group (n=8). **(C and D)** Mechanical **(C)** and thermal **(D)** withdrawal threshold in the Sham, CIBP, CIBP + NC-LV, CIBP + RNAi-LV group (n=16). Compared with Sham group, \* $P < 0.05$ , \*\* $P < 0.01$ ; compared with CIBP group, # $P < 0.05$ .



**Figure 4** NOX4 knockdown alleviates oxidative stress in spinal dorsal horn tissue. **(A)** H<sub>2</sub>O<sub>2</sub> levels in the dorsal horn tissues of Sham, CIBP, CIBP + NC-LV, CIBP + RNAi-LV group (n=8). **(B)** Immunoblot showing expression levels of nNOS and SOD in dorsal horn tissue of Sham, CIBP, CIBP + NC-LV, CIBP + RNAi-LV group (n=8). Compared with Sham group, \* $P < 0.05$ ; compared with CIBP group, # $P < 0.05$ .

with NO to form nitrate, which inactivates SOD. In nerve tissues, NO is mainly produced by neuronal nitric oxide synthase (nNOS), which also plays an important role in neuropathic pain.<sup>15</sup> However, NO is difficult to detect in

tissues and cells. Nevertheless, we found that down-regulation of NOX4 expression significantly inhibited nNOS in dorsal horn tissues (Figure 4B). Taken together, NOX4 promotes oxidative stress in the dorsal horn

microglia by enhancing ROS production and inhibiting SOD, which can be reversed by NOX4 knockdown.

### NOX4 Knockdown Alters Neurotransmitter Receptor Activation

Since ROS accumulation can alter the activation status of the glutamate and gamma-aminobutyric acid (GABA) neurotransmitter receptors, we next analyzed the effect of NOX4 knockdown on these receptors. NR2D, the major subtype of the excitatory receptor NMDAR, was significantly elevated in the rats with CIBP, whereas the inhibitory receptor GABA<sub>A</sub>-γ2 was significantly decreased. Down-regulation of NOX4 reduced the activation levels of NR2D and significantly increased that of GABA<sub>A</sub>-γ2 (Figure 5A). Similar results were seen with the mRNA and total protein expression levels of both receptors in the CIBP and CIBP + RNAi-LV group (Figure 5B and C).

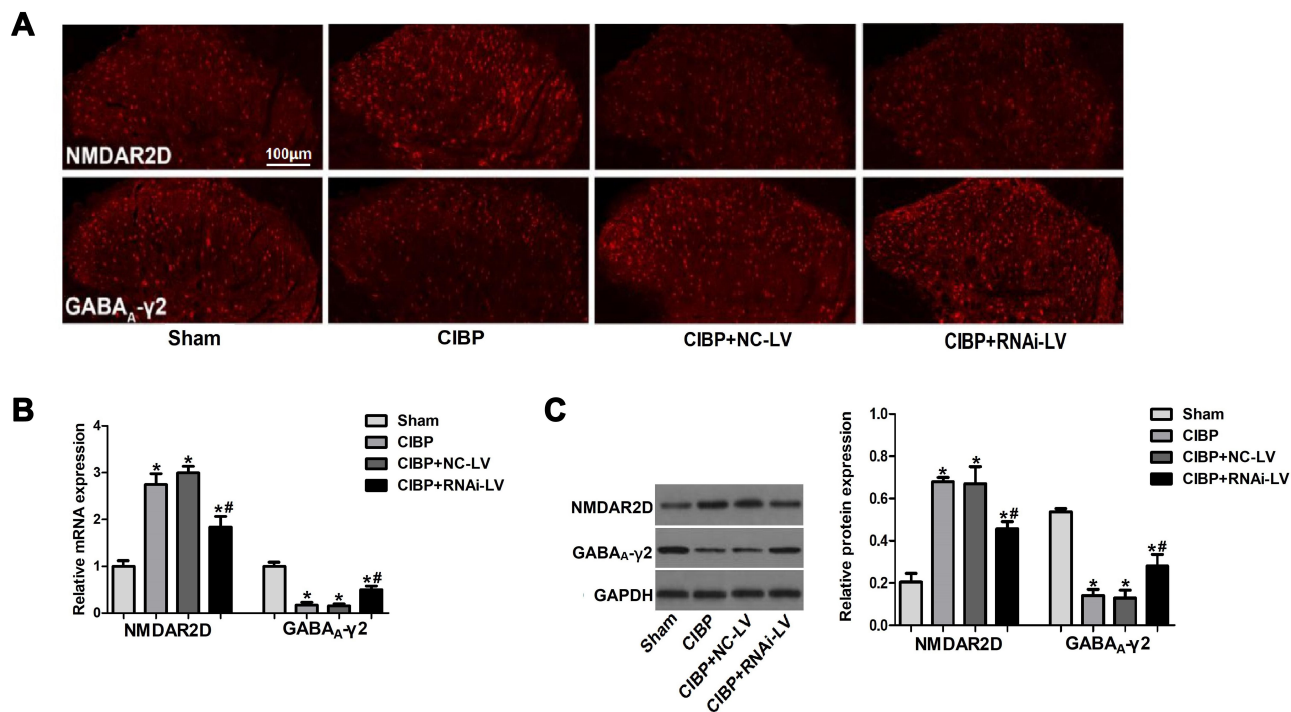
### Discussion

In our study, carcinoma cell inoculation, leading to significant hyperpathia, elevated H<sub>2</sub>O<sub>2</sub> and Nox4 and decreased SOD and levels in the spinal dorsal horn.

These alterations were substantially reversed by intrathecal injection of lentivirus knockdown NOX4.

CIBP is generally the earliest palpable symptom in various malignancies, and most patients seeking treatment for the pain are already in the advanced stages of cancer. However, the pathogenesis of CIBP is complex and ambiguous, leading to unsatisfactory clinical outcomes. Therefore, it is essential to identify the molecular mechanisms underlying CIBP in order to improve treatment strategies.<sup>17</sup>

Peripheral sensitization due to continuous stimulation by various inflammatory cytokines, the acidic tumor-bone microenvironment and altered ROS levels, and central sensitization caused by synaptic plasticity are closely related to the occurrence of CIBP.<sup>18–20</sup> Direct injection of cancer cells into the bone marrow cavity can simulate the clinical characteristics of CIBP and therefore help explore its molecular mechanisms. Tibia injection of rat breast cancer cells into the tibia marrow cavity of adult female rats resulted in mechanical and thermal hyperalgesia within a week, and increased pain sensitivity over a period of 3 weeks. This corresponded to progressive deterioration of the bone trabeculae and the gradual



**Figure 5** The effect of NOX4 knockdown on the activation of neurotransmitter receptors in spinal dorsal horn. (A) Representative immunofluorescence images showing in situ expression of NMDAR2D and GABA<sub>A</sub>-γ2 in the dorsal horn tissue of Sham, CIBP, CIBP + NC-LV, CIBP + RNAi-LV group (n=8). (B) NMDAR2D and GABA<sub>A</sub>-γ2 mRNA levels in Sham, CIBP, CIBP + NC-LV, CIBP + RNAi-LV group (n=8). (C) Immunoblot showing NMDAR2D and GABA<sub>A</sub>-γ2 protein levels in Sham, CIBP, CIBP + NC-LV, CIBP + RNAi-LV group (n=8). Compared with Sham group, \*P <0.05; compared with CIBP group, \*\*P <0.05.



colonization of the marrow cavity with highly differentiated tumor cells. These results confirmed that CIBP was successfully established in the animal model.

The NOX family of proteases are the major catalysts of ROS generation and oxidative stress, and the ROS produced by NOX4 is mainly H<sub>2</sub>O<sub>2</sub>. In this study, we detected a significant upregulation of NOX4 mRNA and protein expression during disease progression. In addition, NOX4 was mainly localized in the microglia of spinal dorsal horn. Microglia are the resident macrophages of the central nervous system (CNS), and account for more than 5% of the nerve cells in the brain. When the CNS is subjected to pessimal stimulation, the microglia are rapidly activated and release effectors like ROS, NO and inflammatory factors (eg, TNF- $\alpha$ , IL-1 $\beta$  and COX-2), which can significantly damage peripheral nerve cells, especially the neurons. Recent studies show that the interaction between neurons and microglia is crucial to central sensitization and the feeling of pain.<sup>19,20</sup> To elucidate the mechanistic role of microglial NOX4 in CIBP, we specifically knocked it down in the dorsal horn, and observed that the animals were significantly sensitized to both mechanical and thermal pain. These preliminary results indicate that NOX4 is a causative factor in CIBP and therefore can be targeted to alleviate bone pain in cancer patients.

The CNS is metabolically demanding due to the high proportion of unsaturated fatty acids, which increases its susceptibility to oxidative stress. The neurons in particular are highly sensitive to ROS-induced injury, which is the mechanistic basis of neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, stroke and other neurological lesions.<sup>21</sup> Hassler et al<sup>10</sup> found that ROS promoted chronic neuralgia after spinal cord injury, and its neutralization significantly relieved the intensity of pain. Little et al<sup>22</sup> have postulated that ROS and NO are key players in the development of pain via central sensitization. H<sub>2</sub>O<sub>2</sub> plays an important role in pain perception and regulation as a diffusive signaling molecule. Indeed, H<sub>2</sub>O<sub>2</sub> levels increased significantly in the dorsal horn of a rat neuropathic pain model established via spinal cord transection and induced neuronal apoptosis through the PI3K/Akt signaling pathway. SOD is closely related to the perception and regulation of pain. Clinical studies show that SOD levels are significantly lower in patients with chronic migraine compared to healthy controls. Consistent with this, the CIBP rats also expressed lower levels of SOD compared to the sham-operated animals. This could be due to the accumulation of large amounts of

ROS that combines with nitric oxide to form peroxynitrite, which in turn reduces the content of SOD through nitrification. In our study, lentivirus knockdown of NOX4 expression can inhibit the increase of NOX4 expression and the increase of H<sub>2</sub>O<sub>2</sub> level induced by CIBP, significantly inhibit the production of nNOS and up-regulate the expression of SOD. This phenomenon may be due to the formation of nitric oxide into peroxide nitrate, which reduces the content of SOD nitrate, while the downregulation of NOX4 weakens the above effects by significantly inhibiting the production of ROS.

Glutamate and  $\gamma$ -aminobutyric acid (GABA) are, respectively, the major excitatory and inhibitory neurotransmitters in the CNS, and play a key role in inflammation-related pain as well as CIBP.<sup>23</sup> The considerable increase in ROS levels in the bone tumor microenvironment leads to the production and secretion of excessive amounts of glutamic acid by the tumor cells. The accumulation of glutamate increases the excitability of afferent nerves by activating N-methyl-D-aspartic acid (NMDA) receptors on peripheral nerve endings, which in turn alters synaptic plasticity in the dorsal horn and enhances pain responsiveness and central sensitization.<sup>23</sup> In fact, direct injection of ROS into the spinal cord of rats with neuropathic pain not only aggravated the pain response but also significantly reduced the expression of GABA. Introducing the ROS scavenger PBN significantly alleviate the above.<sup>24</sup> Consistent with this, our study demonstrated that knocking down NOX4 in the CIBP model significantly downregulated the excitatory receptor NMDAR in the dorsal horn and upregulated the inhibitory receptor GABA<sub>A</sub>- $\gamma$ 2. Other effects of NOX4 on CIBP pathogenesis (eg, peripheral sensitization) have not been further explored in this study. And as reported in the above literatures, ROS plays an important role in the central sensitization of CIBP, and various factors can lead to the increase of ROS level in the spinal dorsal horn, it remains to be further explored whether the reduction of NOX4 can inhibit the production of ROS induced by various pathways.

## Conclusion

NOX4 is significantly upregulated in the spinal dorsal horn microglia of rats with CIBP, and its inactivation alleviated pain by reducing oxidative stress and weakening spinal cord sensitization. Thus, NOX4 is a potential therapeutic target in CIBP and its role needs to be investigated further, along with the additional mechanisms of CIBP.

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## Disclosure

The authors declare that they have no conflict of interests.

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