Low-affinity neurotrophin receptor p75 of brain-derived neurotrophic factor contributes to cancer-induced bone pain by upregulating mTOR signaling

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Abstract. Crucial to the development and maintenance of pain sensations is neurotrophin receptor p75 ($p75^{NTR}$), the low affinity receptor of brain-derived neurotrophic factor (BDNF). This receptor is widespread among dorsal root ganglion (DRG) neurons and the spinal cord. Few reports have demonstrated the specific role of $p75^{NTR}$ in the development of cancer-induced bone pain (CIBP). Therefore the present study examined whether $p75^{NTR}$ contributed to CIBP by upregulating mammalian target of rapamycin (mTOR) signaling. A CIBP rat model was induced and reverse transcription-quantitative polymerase chain reaction was employed to determine $p75^{NTR}$ and mTOR mRNA expression. Immunofluorescence analysis was performed to determine the coexpression of $p75^{NTR}$ and mTOR in DRG neurons, as well as the spinal cord. Von Frey filaments were used to measure the 50% likelihood of paw withdrawal thresholds (PWTs). Spontaneous pain was assessed by ambulatory score. The results demonstrated that compared with the control group, mTOR activation in primary cultured DRG neurons was significantly increased. In addition, mTOR and $p75^{NTR}$ expression was significantly enhanced in the BDNF-treated primary DRG in the BDNF group. *In vivo* experiments determined that mTOR and $p75^{NTR}$ levels were increased in the CIBP rats compared with the sham group. PWT, in response to mechanical stimulation, was significantly lower compared with that in sham rats and the ambulatory score was significantly higher than that in sham rats. Finally, intrathecal injection of a $p75^{NTR}$-targeting small interfering RNA significantly decreased mTOR and $p75^{NTR}$ expression levels in DRG neurons and the spinal cord of CIBP rats, as well as partially reversing the decline in PWTs and the increase in ambulatory score. In conclusion, the present study determined that the activation of BDNF/$p75^{NTR}$/mTOR signaling may participate in nociceptive transmission in CIBP, suggesting a novel mechanism and potential therapeutic target for CIBP treatment and management.

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Introduction

Cancer-induced bone pain (CIBP), resulting from metastasis of primary tumors or bone tumors, is one of the most severe types of chronic pain, and involves a complex molecular mechanism (1,2). CIBP has been demonstrated to cause depression, anxiety and many other complications that can significantly lower quality of life (3). Therefore, investigation into the potential molecular mechanisms underlying CIBP is necessary for the development of novel therapeutic targets.

Brain-derived neurotrophic factor (BDNF) levels are significantly enhanced in the spinal dorsal horn (4) and dorsal root ganglion (DRG) of CIBP rodent models (5). In addition, our previous work demonstrated that BDNF participates in the development and maintenance of behavioral hyperalgesia in the CIBP rat model (6). However, the mechanisms of BDNF signaling that underlie CIBP remain poorly understood. Upregulation of neurotrophin receptor p75 ($p75^{NTR}$), a low-affinity receptor of BDNF, induces a variety of cellular events, triggering several potential proapoptotic cascades. Studies have demonstrated that $p75^{NTR}$ expression is markedly increased in both the peripheral (7) and central nervous systems following noxious stimuli or injury (8). Injury-induced neuropathic pain is significantly reduced via pharmacological blockade of $p75^{NTR}$ in DRG neurons of rats with spinal nerve ligation (7). Mice lacking $p75^{NTR}$ are insensitive to noxious thermal stimuli (9). Despite the widespread expression of $p75^{NTR}$ in DRG and the spinal cord, there are few reports demonstrating that $p75^{NTR}$ is involved in CIBP at the spinal cord level. Furthermore, investigation into the role of BDNF/$p75^{NTR}$ in the pathobiology of cancer-related pain is still at the relatively early stages.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that exists in the mammalian nervous system. There is evidence that widespread dysregulation of mTOR and its downstream signaling pathways contributes to neuropathic pain (10-12). The mTOR pathway is activated in DRG and the spinal dorsal horn in models of CIBP, and intrathecal injection

of rapamycin, a specific inhibitor of mTOR, reduces mechanical hyperalgesia in CIBP rats (13). Finally, it has been demonstrated that activation of mTOR and its downstream effectors, such as ribosomal protein S6 kinase B1 (P70S6K), in DRG and the spinal dorsal horn serves a role in CIBP (14).

The present study hypothesized that the BDNF/p75^{NTR} pathway was involved in the development and maintenance of hyperalgesia in CIBP through mTOR upregulation. Firstly, it was investigated whether mTOR activation in primary sensory neurons was caused by the enhanced release of BDNF. It was then determined whether CIBP increased p75^{NTR} and mTOR expression in DRG and the spinal dorsal horn *in vivo*. Finally, the effect of p75^{NTR} silencing on the development and maintenance of CIBP was determined.

Materials and methods

Animal study. The study was approved by the Ethics Committee of Soochow University. A total of 67 healthy adult female Sprague-Dawley (SD) rats (age, 6-8 weeks; 200±20 g) and 40 healthy neonatal rats (aged 24-48 h; 6.3±1.4 g; neonatal rats of both sexes were used) were kept under controlled conditions (20-25˚C; relative humidity 40-60%; 12 h light/dark cycle with food and water *ad libitum*). Care and handling of rats complied with guidelines of the Institutional Animal Care and Use Committee of Soochow University. All experiments were performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals (15) and the guidelines of the International Association for the Study of Pain (16).

Primary DRG cell culture preparation. Rat DRG cultures were prepared as described previously (17). The neonatal rats were anesthetized with isoflurane and euthanized by cervical dislocation, followed by decapitation. DRG were removed under aseptic conditions and immediately placed in Neurobasal™-A medium (cat. no. 10888022; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.). DRG were digested with collagenase D (1.8-2.0 mg/ml; cat. no. 11088858001; Roche Diagnostics) and 0.25% trypsin (cat. no. T4799; Sigma-Aldrich; Merck KGaA) in D-Hanks' solution at 37˚C for 20 min. The DRG suspensions were centrifuged at 105 x g for 5 min at room temperature. The supernatants were discarded and the DRG were triturated using flame-polished glass pipettes. Dissociated DRG neurons were cultured in 24-well clusters at 37˚C with 5% CO_2 for 24 h, then maintained in Neurobasal-A medium containing 10% FBS. The neurobasal media was replaced after 24 h.

Cell preparation and CIBP rat model. The Walker-256 mammary gland carcinoma cell line was purchased from the American Type Culture Collection and cultured as previously described (3). A total of 10 SD rats were used for the isolation of ascitic fluid in the present study. In brief, Walker-256 cells (2x10^7 cells in 1 ml D-Hank's balanced salt solution; cat. no. PB180321; Beijing Solarbio Science & Technology Co., Ltd.) were injected into the abdominal cavity of SD rats. After 7 days, cancerous ascitic fluid was collected from the rats. Cells were then diluted to a final concentration of 2x10^7 cells/ml with D-Hank's solution. Establishment of the CIBP rat model was in accordance with our previous study (18). Animals were anesthetized via an intraperitoneal injection of sodium pentobarbital (50 mg/kg) for surgery. Then, 20 μl diluted tumor cells were slowly injected into the rat tibial bone marrow cavity with a 23-gauge needle. The sham rat group received 20 μl D-Hank's solution injected into the tibial bone marrow cavity.

Immunofluorescence staining. For the *in vitro* experiments, following 48 h of culture, DRG neurons were treated with exogenous BDNF (20 ng/ml; cat. no. B-250; Alomone Labs) for another 24 h, with the control group cultured in medium only (n=5 for each group). DRG neurons were plated for fluorescent labeling at 1x10^5 cells/well on a cover slip coated with poly-l-lysine. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature followed by three washes in PBS. A blocking step was performed by incubating the coverslips in PBS containing 5% donkey serum (cat. no. ab7475; Abcam) at room temperature for 2 h. For immunofluorescence staining, DRG neurons were incubated with primary antibody at 4˚C overnight: Rabbit anti-mTOR (cat. no. ab2732; 1:200; Abcam); mouse anti-RNA binding fox-1 homolog 3 (NeuN; cat. no. MAB377; 1:500; EMD Millipore); or mouse anti-p75^{NTR} (cat. no. ab61425; 1:100; Abcam). Following primary incubation, samples were then incubated with Alexa Fluor 488-conjugated secondary antibodies (cat. no. ab150077; 1:200; Abcam) or Alexa Fluor 594-conjugated secondary antibodies (cat. no. ab150120; 1:200; Abcam) for 2 h at room temperature. Immunofluorescent staining for mTOR was primarily in the cytoplasm, whilst NeuN staining was mainly in the nucleus of the DRG neurons (11,19). Images were captured using a 20X objective under a fluorescence microscope (Nikon Corporation) and analyzed with Image Pro Plus 6.0 software (Media Cybernetics, Inc.).

For the *in vivo* experiments, the rats were divided into the sham group, CIBP group or CIBP + si-p75^{NTR} group at random (n=4 for each group). Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and transcardially perfused with normal saline followed by 4% paraformaldehyde, as previously described (20). L4-L6 segments of the spinal cord and DRG were removed and fixed for 4 h at 4˚C, then treated with 30% sucrose in PBS at 4˚C overnight. Transverse spinal cord (30 μm) and DRG (12 μm) slices were cut in the cryostat. All slices were blocked with 5% donkey serum at room temperature for 2 h, then incubated with primary antibody, rabbit anti-mTOR (cat. no. ab2732; 1:200; Abcam) and mouse anti-p75^{NTR} (cat. no. ab61425; 1:100; Abcam), at 4˚C overnight. Following primary incubation, samples were incubated with secondary antibodies harboring Alexa Fluor 488 (cat. no. ab150077; 1:200; Abcam) or Alexa Fluor 594 (cat. no. ab150120; 1:200; Abcam) for 2 h at room temperature. Images were captured with a 20X objective under a fluorescence microscope (Nikon Corporation) and analyzed with Image Pro Plus 6.0 software (Media Cybernetics, Inc.).

Intrathecal catheter. According to a method described previously (18,21,22), intrathecal catheters were administered 5 days before the establishment of the CIBP rat model. Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and polyethylene catheters

(PE-10 tube; Smiths Medical) were inserted into the subarachnoid space of the spinal cord between the L4 and L5 spinous processes. Correct positioning was confirmed by the tail flick response immediately following insertion of the catheter. Following housing for 4 days, animals that were observed to have motor dysfunction were excluded from the experiment (22).

Administration of small interfering RNA (siRNA). siRNA sequences targeting the $P75^{NTR}$ gene (GenBank: NM_012610; https://www.ncbi.nlm.nih.gov/genbank/) were purchased from Shanghai GenePharma Co., Ltd. and designed as follows: Sense sequence, 5'-AACGCTTGATGCCCTTTTAGC-3' and antisense sequence, 5'-AATCGCATGCGTTCCATT TCG-3'. The negative sequences, as mismatch controls, were as follows: Sense sequence, 5'-UUCUCCGAACGUGUCACG UTT-3' and antisense sequence, 5'-ACGUGACACGUUCGG AGAATT-3'. Following transfection with siRNA/mismatch using Lipofectamine™ 2000 (also used as the vehicle treatment) (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h or 72 h, the cultured DRG neurons were harvested and used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR). *In vivo* administration was used to investigate whether intrathecal $p75^{NTR}$-targeting siRNA (si-$p75^{NTR}$) could attenuate the mechanical pain of CIBP rats. siRNA was then modified with one stabilizing 2'-fluorodeoxyuridine substitution at the 3'-end of the sense strand. A total of 10 μl si-$p75^{NTR}$/mismatch siRNA/*in vivo*-jetPEI™ (cat. no. 201-10G; Polyplus transfection; Tamar Laboratory Supplies, Ltd.; defined as the vehicle) was injected into the lumbar region of the spinal cord through the intrathecal catheter. Three days after injection of carcinoma cells, the si-p75NTR or corresponding negative controls were injected to the rats once daily for three consecutive days, from days 4 to 6.

RT-qPCR. CO_2 euthanasia followed by physical examination was performed on the animals before collecting the tissues. Total RNA from primary cultured DRG cells, L4-6 spinal cord segments and DRG in *vivo* (n=5 for each group) were extracted using TRIzol® reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (23). Absorbance at 260 and 280 nm was measured for RNA quantification and quality control. RNA samples were reverse transcribed using the RevertAid First Strand cDNA Synthesis kit, according to the manufacturer's protocol (cat. no. K1622; Thermo Fisher Scientific, Inc.). qPCR was performed using SYBR Green PCR MasterMix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The 20 μl reaction volume contained 2 μl cDNA, 1 μl of each pair of primers and 13 μl SYBR Green MasterMix and 3 μl ddH_2O. Thermal cycling was performed as follows: 95˚C for 5 min, 40 cycles of 95˚C for 10 sec, 58˚C for 15 sec and 72˚C for 20 sec. Relative expression levels of each gene were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to β-actin. All experiments were repeated three times for reproducibility. The primer sequences were as follows: mTOR (cat. no. NM_019906) forward, 5'-AGAACCTGGCTCAAG TACGC-3' and reverse, 5'-AGGATGGTCAAGTTGCCG AG-3'; p75NTR (cat. no. NM_012610) forward, 5'-AGGGAT GGCGTGACTTTC-3' and reverse, 5'-GTTGGCTTCAGG CTTATGC-3'; and β-actin (cat. no. NM_031144) forward, 5'-TCTATCCTGGCCTCACTGTC-3' and reverse, 5'-AAC GCAGCTCAGTAACAGTCC-3'.

Behavioral test. The rats were divided into the sham, CIBP, CIBP + si-$p75^{NTR}$, CIBP + mismatch RNA and CIBP + vehicle groups (n=8 for each group). All behavioral tests were performed between 9:00 a.m. and 6:00 p.m. To quantify the mechanical sensitivity of the hind paw, the animals were housed in individual plastic cages for at least 30 min until calm. For mechanical allodynia, von Frey filaments were used to measure the 50% likelihood of paw withdrawal thresholds (PWTs) via the up-and-down method, as described previously (18,24). Spontaneous pain was assessed by ambulatory score in the tumor-injected hind limb, as described previously (24-26). Limb use during spontaneous ambulation was scored on a scale of 0-4: 0, normal use; 1, slight limping; 2, pronounced limping; 3, partial non-use of the limb; and 4, complete lack of limb use. All behavioral tests were performed by an investigator who was blinded to the experimental design.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 5.01. All data are expressed as the mean ± SEM from 4-6 repeats as previously described (27,28). One-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test was used for the analysis of the RT-qPCR and immunofluorescence data. Two-way ANOVA with repeated measures, followed by the Bonferroni post hoc test, was used for PWT and the ambulatory pain score. Student's t-test was used if only two groups were being analyzed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with exogenous BDNF upregulates mTOR and NeuN expression in cultured primary DRG neurons. To investigate whether the mTOR activation in primary sensory neurons was caused by enhanced release of BDNF from microglia, cultured primary DRG neurons were incubated with exogenous BDNF (20 ng/ml) for 24 h, with the dose of BDNF the same as that used in a previous study (29). Following culture for 48 h, DRG neurons of the BDNF group were treated with exogenous BDNF for 24 h whilst the control group was cultured in medium only (without BDNF). DRG neurons were stained for mTOR (Fig. 1A; green; cytoplasm) and NeuN (Fig. 1A; red; nucleus). When compared with the control group, BDNF treatment increased the coexpression of mTOR and NeuN (Fig. 1B; $P<0.01$). In the control group, 19.93±0.02% of NeuN-positive neurons in cultured primary DRG neurons were positive for mTOR, compared with 51.10±0.05% in the BDNF group (Fig. 1B). This result indicated that treatment with BDNF upregulated mTOR in the DRG neurons.

Treatment with exogenous BDNF upregulates the coexpression of mTOR and $p75^{NTR}$ in cultured primary DRG neurons. The effect of BDNF (20 ng/ml) treatment on mTOR and $p75^{NTR}$ expression in DRG neurons was then investigated. DRG neurons were stained for mTOR (Fig. 2A; green; cytoplasm) and $p75^{NTR}$ (Fig. 2A; red; cytoplasm). The area of mTOR immunoreactivity in the control group was 2.92±0.21% and in BDNF group was 6.48±0.41% (Fig. 2B). The area of $p75^{NTR}$

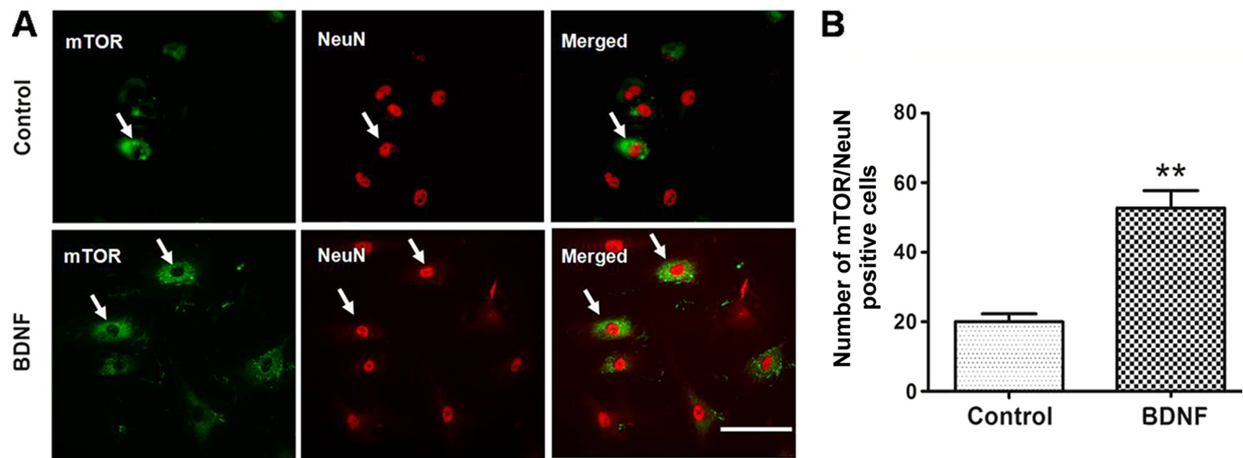

Figure 1. Exogenous BDNF upregulates the coexpression of mTOR and NeuN in cultured primary DRG neurons. (A) Fluorescent micrographs demonstrating that mTOR (green; cytoplasm) and NeuN (red; nucleus) staining was significantly enhanced in cultured DRG neurons following BDNF treatment. (B) Bar graph demonstrating the percentage of mTOR-positive neurons. Scale bar, 100 μm. **P<0.01 vs. control. BDNF, brain derived neurotrophic factor; mTOR, mammalian target of rapamycin; NeuN, RNA binding fox-1 homolog 3; DRG, dorsal root ganglion.

immunoreactivity in the control group was 4.07±0.22% and in BDNF group was 7.79±0.44% (Fig. 2C). The area of mTOR and p75^{NTR} coexpression immunoreactivity was significantly increased in the BDNF group (Fig. 2D; P<0.01). The area of coexpression in the control group was 1.62±0.21% compared with 4.73±0.62% in the BDNF group.

Identification of siRNA for knockdown of p75NTR in cultured primary DRG neurons. RT-qPCR was used to check the transfection efficiency of the siRNA sequences in the primary DRG neurons *in vitro*. Following transfection for 24 h or 72 h, p75^{NTR} mRNA levels in cultured primary DRG neurons transfected with si-p75^{NTR} -24 h (Fig. 3A) or -72 h (Fig. 3B) were significantly reduced when compared with the control, vehicle or mismatch group.

Treatment with si-p75^{NTR} decreases mTOR mRNA expression in the DRG and spinal cords of CIBP rats. The present study subsequently investigated whether the injection of si-p75^{NTR} reversed mTOR mRNA expression in the model of CIBP rats. The sham group rats received 20 μl D-Hanks' solution injection into the tibial bone marrow cavity. The CIBP group rats were injected with 20 μl diluted tumor cells into the rat bone marrow cavity. The CIBP+si-p75^{NTR} group rats were injected with si-p75^{NTR} daily for 3 consecutive days from day 4 to day 6 post-tumor cell injection. It was determined that si-p75^{NTR} treatment markedly suppressed mTOR mRNA expression in DRG of CIBP+ si-p75^{NTR} group rats at day 9 (Fig. 4A). Similarly, mTOR expression levels were significantly decreased in spinal cords of CIBP+ si-p75^{NTR} group rats (Fig. 4B).

Treatment with si-p75^{NTR} reverses upregulated coexpression of mTOR and p75NTR in DRG of CIBP rats. The results indicated that mTOR and p75^{NTR} may participate in the development of chronic pain, including CIBP. It was then investigated whether p75^{NTR} contributed to CIBP by activating mTOR in the DRG. The results demonstrated that the area of p75^{NTR} immunoreactivity in the CIBP group was increased in the DRG slices when compared with that in sham rats. However, following injection with si-p75^{NTR}, the area of p75^{NTR} immunoreactivity in the CIBP+si-p75^{NTR} groups was significantly decreased when compared with that in CIBP rats (Fig. 5A). The area of p75^{NTR} immunoreactivity in the sham group was 15.25±0.46%, 37.86±0.98% in the CIBP group and 11.75±0.82% in CIBP+si-p75^{NTR} groups (Fig. 5B). When compared with the CIBP group, si-p75^{NTR} treatment markedly suppressed mTOR immunoreactivity in CIBP+si-p75^{NTR} group rats following injection with tumor cells (Fig. 5A). The area of mTOR immunoreactivity in the sham group was 25.40±0.40%, 36.06±2.54% in the CIBP group and 19.68±0.75 in CIBP+si-p75^{NTR} group (Fig. 5C). In addition, it was identified that treatment with si-p75^{NTR} reversed the area of coexpression of mTOR and p75^{NTR} in DRG of CIBP rats. The coexpression area of mTOR and p75^{NTR} immunoreactivity in the sham group was 7.04±0.29%, in the CIBP group it was 20.03±0.51%, and in the CIBP+si-p75^{NTR} group it was 5.75±0.21% (Fig. 5D).

Treatment with si-p75^{NTR} reverses mTOR and p75NTR upregulation in the spinal dorsal horn of CIBP rats. The rats were divided into the sham, CIBP or CIBP+si-p75^{NTR} groups at random (n=4). The results demonstrated that the area of p75^{NTR} immunoreactivity in the CIBP group was significantly increased in the spinal dorsal horn slices when compared with that in sham rats. However, following injection with siRNA-p75^{NTR}, the area of p75^{NTR} immunoreactivity in the CIBP+si-p75^{NTR} group was significantly decreased when compared with that in CIBP rats (Fig. 6A). The area of p75^{NTR} immunoreactivity in the sham group was 6.0±0.52%, in CIBP group it was 28.02±2.82%, and in the si-p75^{NTR} group it was 7.29±0.55% (Fig. 6B). When compared with the CIBP group, si-p75^{NTR} treatment significantly suppressed mTOR immunoreactivity in CIBP+si-p75^{NTR} group rats following tumor cell injection. The area of mTOR immunoreactivity in the sham group was 12.28±0.78%, in the CIBP group it was 35.91±2.53%, and in the CIBP+si-p75^{NTR} group it was 29.53±1.48% (Fig. 6C). In addition, it was identified that treatment with siRNA-p75^{NTR} reversed the effect on the area of mTOR and p75^{NTR} coexpression in the spinal dorsal horn

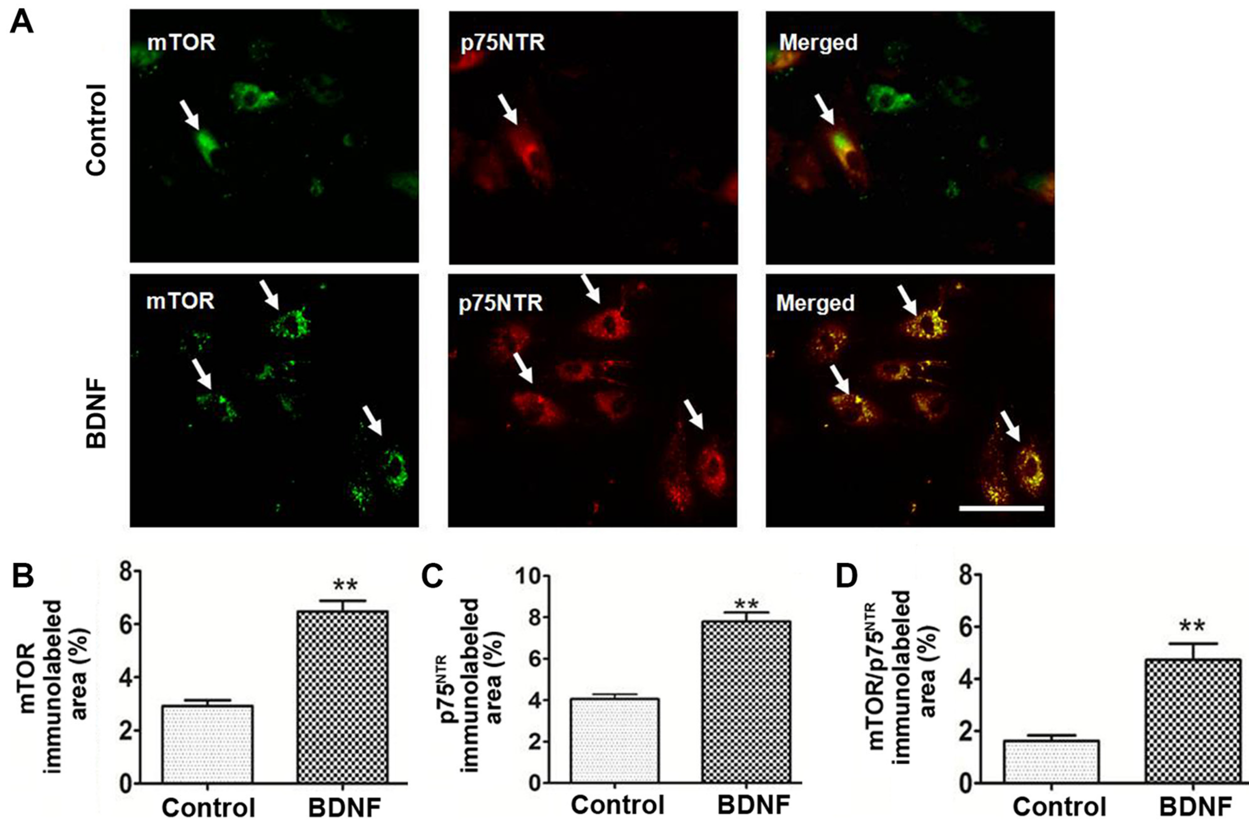

Figure 2. Exogenous BDNF upregulates the coexpression of mTOR and $p75^{NTR}$ in cultured primary DRG neurons. (A) Fluorescent micrographs demonstrating that BDNF treatment (20 ng/ml) significantly enhanced the coexpression of mTOR (green) and $p75^{NTR}$ (red). (B) Bar graph quantifying the immunolabeled area of mTOR. (C) Bar graph quantifying the immunolabeled area of $p75^{NTR}$. (D) Bar graph quantifying the immunolabeled area of coexpression of mTOR and $p75^{NTR}$. Scale bar, 100 μm. **P<0.01 vs. control. BDNF, brain derived neurotrophic factor; mTOR, mammalian target of rapamycin; $p75^{NTR}$, neurotrophin receptor p75; DRG, dorsal root ganglion.

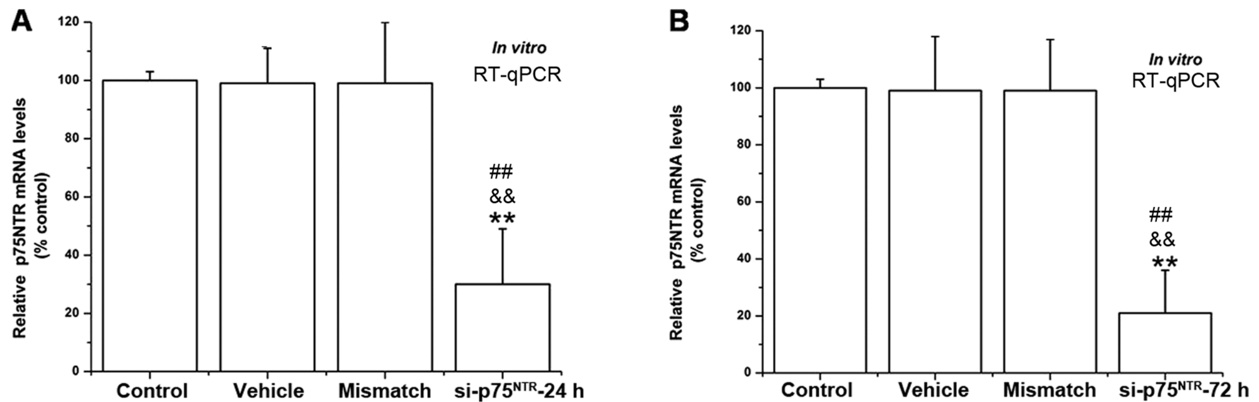

Figure 3. Identification of siRNA for knockdown of $p75^{NTR}$ in cultured primary DRG neurons. (A) Transfection of si-$p75^{NTR}$ for 24 h significantly reduced the $p75^{NTR}$ mRNA level in cultured rat DRG neurons. (B) Transfection of si-$p75^{NTR}$ for 72 h significantly reduced the $p75^{NTR}$ mRNA level in cultured DRG neurons. **P<0.01 vs. control; $^{\&\&}$P<0.01 vs. vehicle group; $^{\#\#}$P<0.01 vs. mismatch group. siRNA, small interfering RNA; $p75^{NTR}$, neurotrophin receptor p75; DRG, dorsal root ganglion; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

of CIBP rats. The coexpression area of mTOR and $p75^{NTR}$ immunoreactivity in the sham group was 2.36±0.17%, in the CIBP group it was 6.49±0.20%, and in the CIBP+si-$p75^{NTR}$ group it was 3.89±0.34% (Fig. 6D).

Treatment with si-$p75^{NTR}$ attenuates hyperalgesia in CIBP rats. Finally, the present study investigated whether blocking $p75^{NTR}$ upregulation in DRG and dorsal horns via intrathecal injection of si-$p75^{NTR}$ decreased the behavioral hyperalgesia of CIBP rats. Compared with the sham group rats, the 50% PWT of CIBP group rats was significantly decreased between days 6 and 18. In the CIBP+si-$p75^{NTR}$ group, the PWT was significantly higher compared with that in the CIBP, CIBP + mismatch RNA and CIBP + vehicle group rats (Fig. 7A). By contrast,

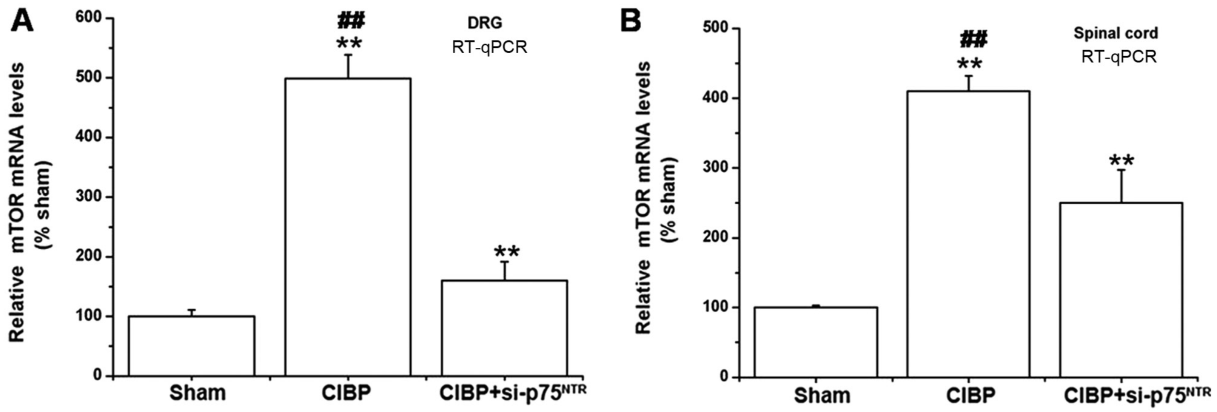

Figure 4. si-p75^{NTR} decreases mTOR mRNA expression in the DRG and spinal cord of CIBP rats. (A) si-p75^{NTR} treatment significantly suppressed mTOR mRNA expression in the DRG of CIBP group rats. (B) si-p75^{NTR} treatment significantly reduced mTOR expression levels in spinal cord of CIBP group rats. **P<0.01 vs. sham group; $^{##}$P<0.01 vs. si-p75^{NTR} group. si, small interfering; p75^{NTR}, neurotrophin receptor p75; mTOR, mammalian target of rapamycin; DRG, dorsal root ganglion; CIBP, cancer-induced bone pain; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

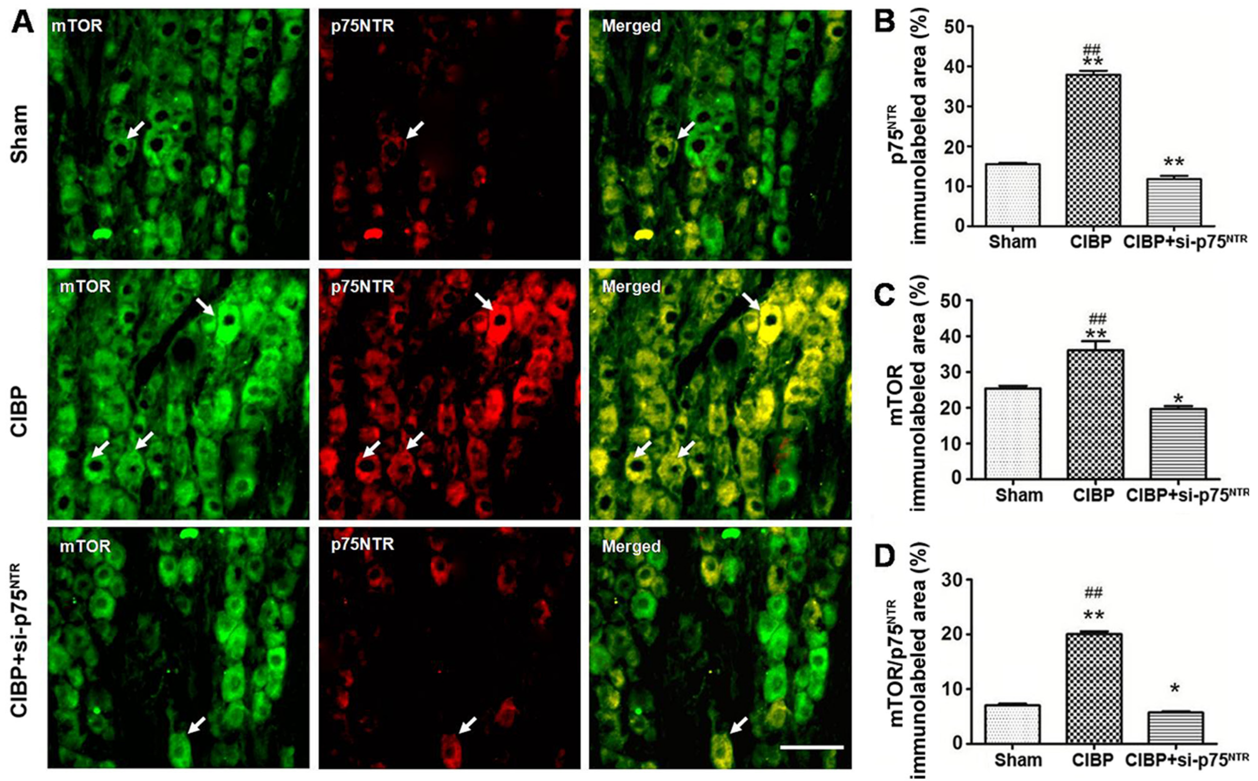

Figure 5. si-p75^{NTR} reverses mTOR and p75^{NTR} upregulation and coexpression in the DRG of CIBP rats. (A) Fluorescent micrographs demonstrating mTOR and p75^{NTR} expression in DRG slices. (B) Bar graph quantifying the immunolabeled area of p75^{NTR}. (C) Bar graph quantifying the immunolabeled area of mTOR. (D) Bar graph quantifying the immunolabeled area of mTOR and p75^{NTR}. Scale bar, 100 μm. *P<0.05 and **P<0.01 vs. sham group.; $^{##}$P<0.01 vs. CIBP+si-p75^{NTR} group. si, small interfering; p75^{NTR}, neurotrophin receptor p75; mTOR, mammalian target of rapamycin; DRG, dorsal root ganglion; CIBP, cancer-induced bone pain.

the ambulatory scores of CIBP + si-p75^{NTR} group rats were significantly lower than those observed in the CIBP, CIBP + mismatch RNA and CIBP + vehicle group rats (Fig. 7B).

Discussion

CIBP is a growing health concern due to the severity of the pain, the limited efficacy of current drug treatments and the unacceptable side effects for cancer patients (30). In order to mimic the clinical pain of patients with cancer-related pain, studies have injected cancer tumor cells into the bone to produce hyperalgesia in animal models (3,18). Emerging evidence has indicated that the BDNF tropomyosin receptor kinase B (TrkB) complex is trafficked to the cell for downstream signaling pathway transduction, and then participates in the development of neuropathic pain (31-33).

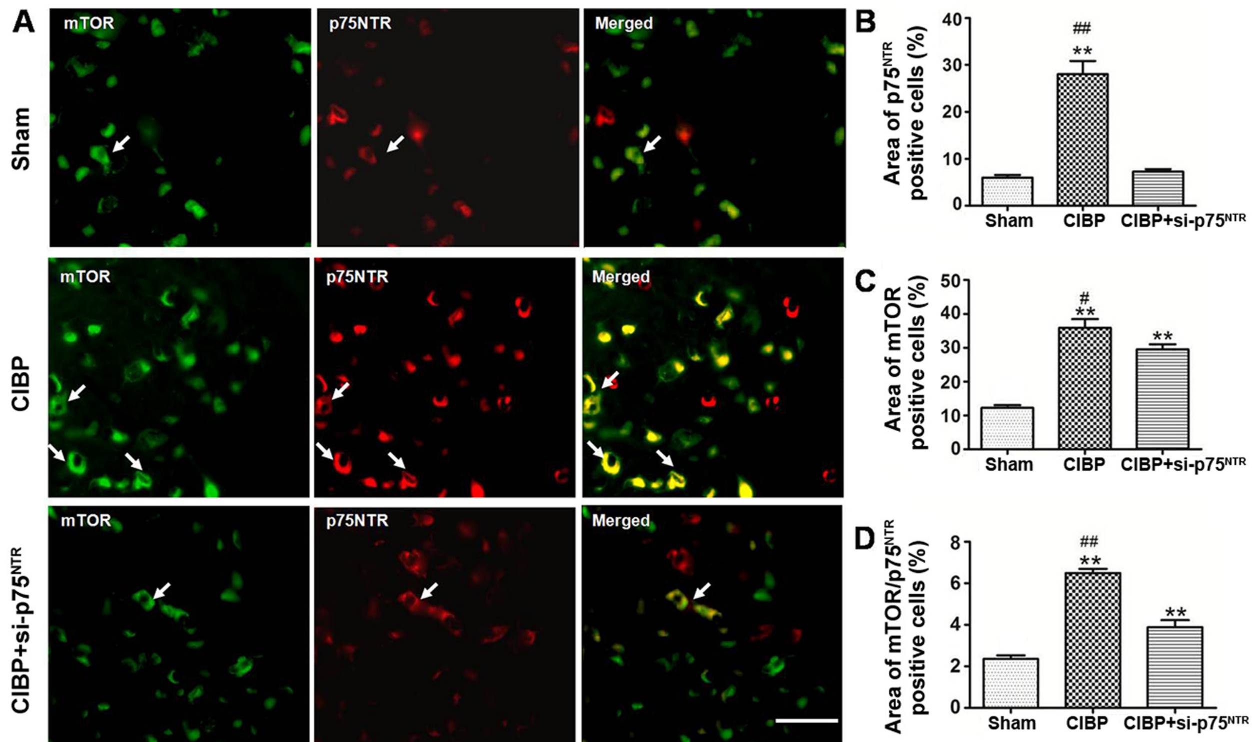

Figure 6. si-p75^{NTR} reverses mTOR and p75^{NTR} coexpression in the spinal cord of CIBP rats. (A) Fluorescent micrographs demonstrating mTOR and p75^{NTR} staining in spinal cord slices. (B) Bar graph quantifying the immunolabeled area of p75^{NTR}. (C) Bar graph quantifying the immunolabeled area of mTOR. (D) Bar graph quantifying the immunolabeled area of mTOR and p75^{NTR} coexpression. Scale bar, 100 μm. **P<0.01 vs. sham group; #P<0.05 and ##P<0.01 vs. CIBP+si-p75^{NTR} group. si, small interfering; p75^{NTR}, neurotrophin receptor p75; mTOR, mammalian target of rapamycin; DRG, dorsal root ganglion; CIBP, cancer-induced bone pain.

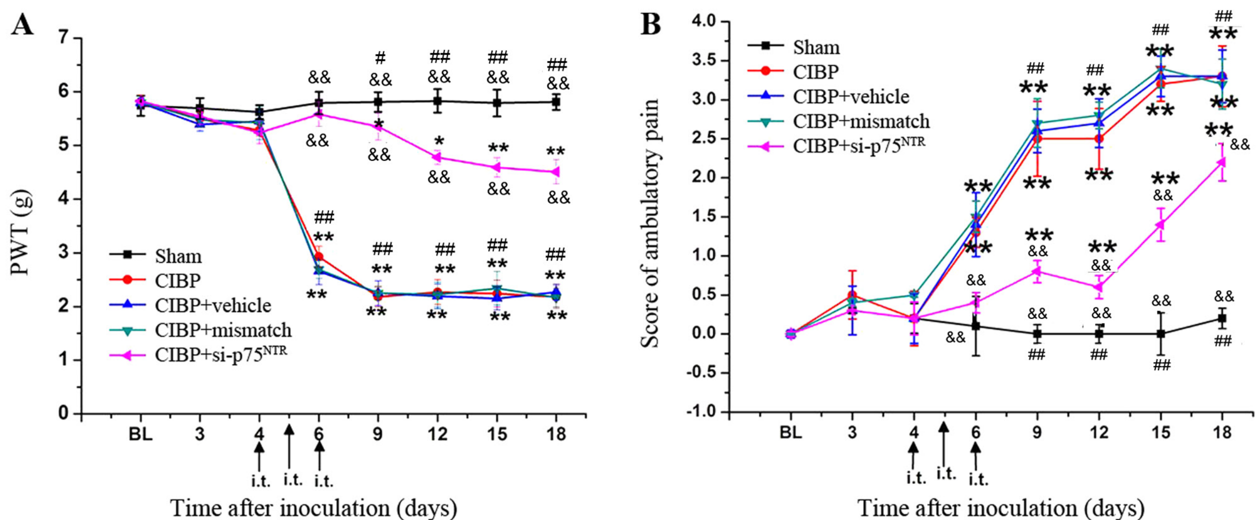

Figure 7. Treatment with si-p75^{NTR} attenuates hyperalgesia in CIBP rats. (A) Following injection with si-p75^{NTR}, the PWTs of CIBP+si-p75^{NTR} group rats were significantly higher compared with those in the CIBP, CIBP+mismatch or CIBP+vehicle groups. (B) Following injection with si-p75^{NTR}, the ambulatory scores of the CIBP+siRNA-p75^{NTR} group were significantly lower than those observed in the CIBP, CIBP+mismatch and CIBP+vehicle groups. No significant differences were observed between CIBP+mismatch, CIBP+vehicle rats and CIBP group rats. *P<0.05 and **P<0.01 vs. sham group; &&P<0.01 vs. CIBP group; #P<0.05 and ##P<0.01 vs. CIBP+si-p75^{NTR} group. si, small interfering; p75^{NTR}, neurotrophin receptor p75; CIBP, cancer-induced bone pain; PWT, paw withdrawal thresholds; i.t., intrathecal injection.

Chodroff *et al* (34) demonstrated that BDNF signaling constitutes a novel mechanism whereby oral squamous cell carcinoma induces pain. Identification of the key role of TrkB signaling in oral cancer pain may serve as a novel target for drug development. Recent research has demonstrated that a population of positive-TrkB sensory neurons are both necessary and sufficient for producing pain from only light touch following nerve injury in mice (35). These results highlighted

the potential value of BDNF TrkB as a therapeutic target. However, another report indicated that blocking of all three Trk receptors markedly inhibited sprouting and neuroma formation in sensory nerve fibers and reversed CIBP behavior by 50-60% (20). It is likely that BDNF might contribute to the development and progression of CIBP by regulating other signaling pathways which need to be further explored. Yao *et al* (36) demonstrated that the neurotrophin receptors p75^{NTR} in the DRG and spinal cords were significantly increased in asparaginyl endopeptidase-induced CIBP. However, this study did not further investigate whether p75^{NTR} contributed to the hypersensitivity of CIBP. The present study determined that the hyperalgesia in CIBP was significantly attenuated through inhibition of the BDNF/p75^{NTR} pathway. Between days 6 and 12, the effect reached a >80% reduction compared with the sham group, which indicated its potency. However, the molecular mechanisms underlying CIBP are complex and remain to be fully understood. The reversion appeared to be attenuated after day 12, indicating that other factors may play a core role at that point.

A previous study determined that treatment with mTOR inhibitors blocks the BDNF-increased metastasis of neuroblastoma (37). In order to investigate whether BDNF contributed to CIBP by upregulating mTOR, the present study used exogenous BDNF to imitate the over-secretion of BDNF by microglia. It was determined that BDNF treatment increased mTOR expression in primary cultured DRG neurons. In addition, it was demonstrated that mTOR mRNA levels were significantly increased in the DRG neurons and spinal dorsal horn of CIBP rats. These results indicated that mTOR activated through the BDNF signaling pathway participated in regulating behavioral hyperalgesia in the CIBP rat model. Research has clarified that CIBP-induced mechanical allodynia and thermal hyperalgesia are markedly attenuated when mTOR is inhibited by intrathecal injection of rapamycin (14,37). The activation of mTOR further triggers the phosphorylation of downstream pathways, such as P70S6K and eukaryotic translation initiation factor 4E binding protein 1, to regulate mRNA translation and protein synthesis (10,14,38). Preclinical and clinical studies have demonstrated that N-methyl-D-aspartate (NMDA) receptor inhibition produces a significant analgesic effect on CIBP, suggesting that NMDA receptors are required for the induction of hypersensitivity (14,29). These results are consistent with the authors' previous study, which demonstrated that BDNF-activated NMDA receptors in the spinal cord or DRG were involved in the development of central sensitization and behavioral hypersensitivity in CIBP rats (18). Shih *et al* (14) further demonstrated that NMDA receptors participate in the activation of mTOR and its downstream signaling pathway in the spinal dorsal horn in a CIBP model (13,14). A recent study demonstrated that BDNF/mTOR signaling could activate the NMDA receptor, which is required for inflammatory pain-related aversion in the rostral anterior cingulate cortex of rats (39). Therefore, the present study speculated that BDNF/p75^{NTR} may be involved in the regulation of hyperalgesia by activating mTOR-NMDA signaling in the CIBP model. However further investigation is required in future work.

In conclusion, the present study identified the critical function of BDNF/p75^{NTR} by upregulating mTOR expression in the DRG and spinal cord of CIBP rats. mTOR expression was significantly reduced in the DRG and spinal cords following p75^{NTR} silencing, and behavioral hyperalgesia in CIBP rats was attenuated. The present findings suggested that BDNF/p75^{NTR}/mTOR signaling may serve a major role in the development of CIBP; therefore, potent and specific inhibitors of p75^{NTR} could be developed into novel drugs for treating CIBP.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XWM performed experiments, analyzed data, prepared figures and drafted the manuscript. XHJ performed experiments, analyzed data and drafted the manuscript. XW performed experiments and analyzed data. LNW designed and supervised the experiments and edited the manuscript. JPY analyzed data and edited the manuscript. FHJ analyzed data and edited the manuscript.

Ethics approval and consent to participate

Experimental protocols were approved by the Institutional Animal Care and Use Committee of Soochow University. All experiments were performed in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals and the guidelines of the International Association for the Study of Pain.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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