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The Spinal $\alpha 7$ -Nicotinic Acetylcholine Receptor Contributes to the Maintenance of Cancer-Induced Bone Pain

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Introduction: Cancer-induced bone pain (CIBP) is acknowledged as a multifactorial chronic pain that tortures advanced cancer patients, but existing treatment strategies for CIBP have not been satisfactory yet. Investigators have demonstrated that the activation of $\alpha 7$ -nAChRs exerts analgesic effects in some chronic pain models. However, the role of spinal $\alpha 7$ -nAChRs in CIBP remains unknown. This study was designed to investigate the role of $\alpha 7$ -nAChRs in a well-established CIBP model induced by Walker 256 rat mammary gland carcinoma cells.

Methods: The paw withdrawal threshold (PWT) of the ipsilateral hind paw was measured using von Frey filament. The expressions of spinal $\alpha 7$ -nAChRs and NF- κ B were measured with Western blotting analysis. Immunofluorescence was employed to detect the expression of $\alpha 7$ -nAChRs and co-expressed of $\alpha 7$ -nAChRs with NeuN or GFAP or Iba1.

Results: Experiment results showed that the expression of spinal $\alpha 7$ -nAChRs was significantly downregulated over time in CIBP rats, and in both CIBP rats and sham rats, most of the $\alpha 7$ -nAChRs located in neurons. Behavioral data suggested PNU-282,987, a selective $\alpha 7$ -nAChRs agonist, dose-dependently produced analgesic effect and positive allosteric modulator could intensify its effects. Further, repeated administration of PNU-282,987 reversed the expression of $\alpha 7$ -nAChRs, inhibited the nuclear factor kappa B (NF- κ B) signaling pathway, and attenuates CIBP-induced mechanical allodynia state as well.

Conclusion: These results suggest that the reduced expression of spinal $\alpha 7$ -nAChRs contributes to the maintenance of CIBP by upregulating NF- κ B expression, which implying a novel pharmacological therapeutic target for the treatment of CIBP.

Keywords: cancer-induced bone pain, $\alpha 7$ -nAChR, NF- κ B, PNU-282,987

Introduction

Cancer-induced bone pain (CIBP) is usually the trickiest for clinicians for its complicated mechanisms, which involved immune cells, nerves and mechano-sensitive receptors.¹⁻³ Statistically, more than 70% of cancer patients suffer pain⁴ in which CIBP is the most common type of pain, and their quality of life is reduced markedly because of poor mood, the ability to walk⁵ and sleep.⁵⁻⁷ The World Health Organization (WHO) guidelines recommend a stepladder approach using non-opioids, weak and then strong opioids for the relief to CIBP,⁸ but the problem is still inadequately addressed, and there remains a great challenge to effectively alleviate the patients' pain because of limited effects and/or intolerable side effects.^{9,10} Given that the existing treatment for CIBP remains deficient according to its known mechanisms, the

specific cellular and molecular mechanisms underlying CIBP remain a pressing need to do more to understand.

Nicotine acetylcholine receptors (nAChRs) exist extensively in the central and peripheral nervous system as well as on immune cells, having been explored as a pain relief strategy in the past three decades. Alpha7-nAChR, one of the nicotinic acetylcholine receptors, is composed of five $\alpha 7$ subunits per receptor, possessing the characteristic of high calcium permeability and rapid desensitization in response to the stimulation from high concentration agonist^{11,12} compared with other nAChRs subtypes. They are reported to be beneficial for retinal diseases,¹³ psychiatric disorders,¹⁴ and some central nervous system disorders.¹⁵ The $\alpha 7$ -nAChR has been shown to be distributed in the pain transmission pathway and evidences are accumulated that $\alpha 7$ -nAChR activation has been related to analgesic effects in some animal models.^{16–21} However, so far, the role of spinal $\alpha 7$ -nAChR in CIBP has not been studied yet. Nuclear factor kappa-B (NF- κ B) is a heterodimer composed of p65 and p50 or p52 subunits. NF- κ B is thought to control many genes related to nociceptive mediators.^{22,23} Accumulating evidence indicates that activation of NF- κ B enhanced pain perception, while inhibition of NF- κ B reduced pain, including cancer-induced bone pain.^{24–26} Lipopolysaccharide activating $\alpha 7$ -nAChR blocked NF- κ B nuclear translocation.²⁷ Here in this study, we tested whether $\alpha 7$ -nAChR and NF- κ B signaling pathways were involved in the maintenance of CIBP and its possible mechanisms.

Materials and Methods

Animals and Ethical Statement

One hundred and forty-three virgin female Sprague–Dawley rats (200–220 g, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) that are highly susceptible to Walker 256 rat mammary gland carcinoma cells (from our laboratory storage and approved by the research ethics committee of Tongji hospital) were used in this study. Sex selection for building CIBP model depends on tumor tissue of origin and epidemiological characteristics of relative cancer between men and women.²⁸ The rats were housed in groups of one to three per cage under controlled conditions ($24 \pm 0.5^\circ\text{C}$, relative humidity was about 55%, 12 h alternating light-dark cycle) and given food and water ad libitum. All experimental protocols were acquired approval from the Institutional Animal Care and Use Committee of

Huazhong University of Science and Technology and conform to guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of Carcinoma Cells

Walker 256 rat mammary gland carcinoma cells were taken out from the liquid nitrogen tank, quickly recovered in a 37°C water bath, and then diluted to 4×10^7 cells/mL. For obtaining more tumor cells quickly, the diluted cells (4×10^7 cells/mL, 1 mL) were inoculated into the abdominal cavity of female SD rats. About 5 to 7 days later, carcinoma cells were harvested from the peritoneal fluid. Then, cells were diluted to 4×10^7 cells/mL with sterile phosphate buffer saline (PBS) and placed on ice for tumor cells implantation (TCI).

Establishment of Tibial CIBP Models

CIBP model was performed as described previously.^{29,30} First of all, under deep anesthesia with sodium pentobarbital (50 mg/kg, i.p.), the skin of the operation area was disinfected and a small incision was made to expose the proximal right tibia. And then, a hole was drilled on the tibia and Walker 256 cells (4×10^7 cells/mL, 10 μL) harvesting from the peritoneal fluid were slowly injected into the tibial hole using a 10 μL Hamilton syringe. The sham group was applied the same operation, but injected with 10 μL PBS instead. The syringe was then detained for at least 2 minutes to ensure adequate diffusion of carcinoma cells in the bone cavity. After that, the hole was sealed with bone wax as soon as the syringe was gotten out. Last of all, the wound was stitched with 3–0 silk thread. Animals were kept warm on a heated pad until recovering from anesthesia. Rats that successfully constructed a cancer-induced pain model not only had changes in the paw withdrawal threshold but also the bone destruction at the tumor injection site; however, the injury caused by NS injection of sham rats healed several days later.

Drug Administration

We modified the previous intrathecal catheterization (i.t.) methods^{31,32} and performed it 7 days before establishing CIBP models. In simple terms, the rats were anesthetized using sodium pentobarbital (50 mg/kg, i.p.), and then a PE-10 polyethylene catheter (PE-10 tube) was implanted into the subarachnoid space from the spinal cord intervertebral space between L5 and L6. If there were no signs of a tail-flick response or cerebrospinal fluid in the catheter when inserting the catheter and a sudden paralysis of both

legs after an i.t. injection of 2% lidocaine (10 μ L), the rats were eliminated. α 7-nAChR agonist PNU-282,987 and α 7-nAChR antagonist Methyllycaconitine citrate (MLA) were purchased from Med Chem Express (Monmouth Junction, NJ, USA), and α 7-nAChR positive allosteric modulator (PAM) PNU-120,596 were purchased from Sigma (Sigma-Aldrich, St. Louis, USA). The first two drugs were dissolved in 0.9% saline and the last one was dissolved in 5% DMSO. The dose of drugs was determined according to previous reports.^{20,33,34} The specific protocol was as follows: for acute treatment, a single dose of PNU-282,987 (0.5, 0.25, 0.1 mg/kg, i.t.) was given on day14 after the TCI; for chronic treatment, PNU-282,987 was given from day 14 to day 18 once daily. MLA (10 μ g/10 μ L, i.t.) or PNU-120,596 (4 μ g/10 μ L, i.t.) was given 30 min before PNU-282,987 (0.5mg/kg, i.t.) injection.

Behavioral Tests

In order to evaluate mechanical allodynia, we stimulated the rats regarding the paw withdrawal threshold (PWT) of the ipsilateral hind paw using von Frey filament as described previously.³⁵ In short, each rat was placed in a single transparent chamber on a metal mesh floor and acclimated to the environment for 20–30 min before behavioral testing. The testing was started at a 2 g filament. A positive response was defined as an abrupt withdrawal, licking, and shaking of the tested paw. Once a positive response happened, the von Frey filament was replaced with a next lower one; otherwise, the next higher one was applied. Finally, the lowest numerical value of von Frey filament eliciting a positive response was recorded as the PWT. All behavioral tests on animals were carried out by a blinded investigator.

Immunofluorescence

Under appropriate anesthetic depth with sodium pentobarbital (50 mg/kg, i.p.), each animal was accepted perfusion transcardially with about 250 mL 4% icy paraformaldehyde (PFA) after 250 mL PBS. The next steps were to harvest L4 to L5 spinal segments, post-fix these samples in 4% PFA, and dehydrate them in 30% sucrose solution overnight at 4°C. After that the tissues were embedded in O.C.T. and sectioned 20 μ m thick in a cryostat (CM1900, Leica, Wetzlar, Germany), last of all, keep the sections at -20°C.

Single Immunostainings

The spinal sections were incubated with 3% Triton X-100 for 15 min and blocked with 5% donkey serum for 1 hour, and then placed at 4°C for a night with rabbit anti- α 7 nAChR antibody (1:50, 21,379-1-AP, Proteintech Group, Wuhan, China). The sections were incubated with IFKine Green Affinipure Donkey Anti-Rabbit IgG (1:100, A24221, Abbkine) for 2 hours at 37°C and required 5 washes (7 minutes each) with PBST. Sections were drained excess PBST and covered the coverslips after adding 50% glycerol. Photomicrographs were captured by a fluorescence microscope (DM2500, Leica, Wetzlar, Germany).

Double-Labeling Procedures

Spinal sections were incubated with a mixture of rabbit anti- α 7 nAChR antibody (1:50, 21,379-1-AP, Proteintech Group, Wuhan, China), and mouse anti-neuronal nuclei (NeuN) antibody (1:200; MAB377; EMD Millipore, Billerica, MA; neuronal marker), mouse anti-gial fibrillary acidic protein (GFAP) antibody (1:200; 3670; Cell Signaling Technology, Danvers, MA; astrocytic marker), or goat anti-ionized calcium-binding adapter molecule 1 (Iba1) antibody (1:200; ab5076; Abcam; microglial marker), respectively. The next day, slices were washed with PBST 5 times, then incubated with a mixture of IFKine Green Affinipure Donkey Anti-Rabbit IgG (1:100, A24221, Abbkine) and IFKine Green Affinipure Donkey Anti-Mouse IgG (1:200, A24411, Abbkine) or IFKine Green Affinipure Donkey Anti-Goat IgG (1:200, A24431, Abbkine) for 2 hours at room temperature. The other steps were the same as the single immunostainings described above.

The number of α 7-nAChRs and double-labeled α 7-nAChRs/NeuN or Iba 1 or GFAP-positive cells in the dorsal horn (laminae I–IV) was counted by Software Image-Pro Plus Version 6.0 for Windows™ (MediaCybernetics, Bethesda, USA). Three slices were randomly selected from each rat for counting. The average value of each rat was used as five independent values for further statistical analysis. Then, we calculated the percentage of positive cells co-expressed with NeuN or GFAP or Iba1 in the total positive cells.

Western Blot Analysis

Western blot analysis was performed to quantify the expression of aimed protein as described previously.³⁶

Under appropriate anesthesia with sodium pentobarbital (50 mg/kg, i.p.), the lumbar enlargements were obtained and homogenized in radio-immunoprecipitation assay (RIPA) lysis buffer containing inhibitor cocktail. The supernatant was kept following centrifugation (16,000×g, 4°C, 15 min, 2 times) and the protein concentration was determined using the Bradford method and standardized. The protein samples were heated for 15 min at 100°C with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. About 40 µg aimed proteins were separated by 10% sodium dodecyl sulfate-PAGE electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes (IPVH00010, EMD Millipore). The membranes were blocked with 5% skim milk in Tris-buffered saline and Tween 20 (TBST, 0.1%) for 2 h at room temperature, and subsequently incubated overnight at 4°C with anti- $\alpha 7$ nAChR antibody (1:1000, 21,379-1-AP, Proteintech Group, Wuhan, China) or p65 (1:1000, 10,745-1-AP, Proteintech Group, Wuhan, China) and rabbit anti-GAPDH antibody (1:5000; AS1039; Aspen), respectively. The protein bands were washed in TBST for 4 times (8 minutes each) and incubated with HRP goat anti-rabbit (1:5000; A21020; Abbkine) for 2 h at 37°C. After that, the protein bands were emerged using Super Lumina ECL Plus HRP Substrate Kit (K22030; Abbkine). Finally, we calculated the mean intensities of selected areas and normalized them to values of GAPDH by Image Lab software (BIO-RAD, Chemi DocXRS1, USA).

Statistical Analysis

All results are presented as the mean standard error of the mean (SEM) and performed by GraphPad Prism version 8. PWTs were analyzed by one-way ANOVA followed by Bonferroni post hoc test; Two-way analysis of variance with repeated measures, followed by Bonferroni post hoc test was used for Western blot, immunochemistry data. NF- κ B Western blot data before drugs administration were analyzed by unpaired *t*-test; $p < 0.05$ was considered statistically significant.

Results

Expression and Cell Localization of $\alpha 7$ -nAChRs in Spinal Cord of CIBP Rats

The experimental design is shown in Figure 1A. Firstly, we tested the expression of $\alpha 7$ -nAChRs on cancer-induced bone pain (CIBP) rats. As expected, mechanical allodynia

was established on Walker 256 tumor cells implanted rats (d7: $F(2,15) = 40.76$, $p < 0.0001$; $t = 6.774$, $p < 0.0001$; d14: $F(2,15) = 40.85$, $p < 0.0001$; $t = 7.46$, $p < 0.0001$; d21: $F(2,15) = 55.02$, $p < 0.0001$; $t = 8.327$, $p < 0.0001$) (Figure 1B). And then the spinal expression of $\alpha 7$ -nAChRs was tested by western-blot. Compared with sham rats, the expression of $\alpha 7$ -nAChRs was significantly decreased on day14 and day21 after tumor cells implantation ($F_{(4,20)} = 10.23$, $p = 0.0001$; $t_{14} = 3.86$, $p = 0.0098$; $t_{21} = 5.099$, $p = 0.0005$) (Figure 1C and D). To figure out cellular localization of $\alpha 7$ -nAChRs in lumbar spinal cord dorsal horn, we used double stain of anti- $\alpha 7$ -nAChRs and anti-neuron marker (NeuN), anti-astrocyte marker (GFAP), and anti-microglia marker (Iba1). As presented in Figure 2A and B, $\alpha 7$ -nAChRs were predominantly colocalized with NeuN, only a few with GFAP and Iba1 in the spinal cord, both in CIBP and sham rats. With the results tallied, it was about 82.5% $\alpha 7$ -nAChRs in sham rats and 75.2% $\alpha 7$ -nAChRs in CIBP rats were expressed in neurons, and less than 5% of $\alpha 7$ -nAChRs were expressed on activated microglia and activated astrocytes, the rest might be expressed on cells such as oligodendrocyte, immune cells, inactive microglia and inactive astrocytes (Figure 2C and D).

Pharmacological Activation of $\alpha 7$ -nAChRs Produced Dose-Dependent Analgesic Effects in CIBP Rats

The experimental design is shown in Figure 3A. To explore the analgesic effect of spinal $\alpha 7$ -nAChRs in CIBP rats by pharmacological methods, firstly, a single dose of $\alpha 7$ -nAChR agonist, PNU-282,987 was intrathecally administrated on day14 after TCI. And the behavioral tests were performed at 0.5, 1, 1.5, 2, 2.5 and 3 h after injection. When compared with the normal saline given rats, 0.5 and 0.25 mg/kg PNU-282,987 treatment notably relieved cancer-induced bone pain, lasting from 0.5 h ($F(4,17) = 12.71$, $P < 0.0001$; $t_{0.5\text{mg/kg}} = 5.801$, $p = 0.0002$; $t_{0.25\text{mg/kg}} = 4.687$, $p = 0.0021$) to 2.5 h ($F(4,17) = 612.71$, $P = 0.0021$; $t_{0.5\text{mg/kg}} = 4.171$, $p = 0.0064$; $t_{0.25\text{mg/kg}} = 3.436$, $p = 0.0315$) (Figure 3B). However, compared with that of the NS group, treatment of 0.1 mg/kg PNU-282,987 had no obvious influence on PWT. When $\alpha 7$ -nAChR antagonist, the MLA (i.t.10µg/10µL) was administrated 30 min before PNU-282,987 (i.t., 0.5mg/kg) injection, the analgesic effect of PNU-282,987 could be totally blocked (0.5 h: $F(4,17) = 12.71$, $P < 0.0001$; $t = 5.139$, $p = 0.0008$; 1 h: $F(4,17) = 15.99$, $P < 0.0001$; $t = 5.975$, $p = 0.0002$; 1.5 h: $F(4,17) = 15.22$, $P < 0.0001$; $t = 5.75$, $p = 0.0002$; 2h: $F(4,17) = 12.02$, $P < 0.0001$; $t = 4.848$,

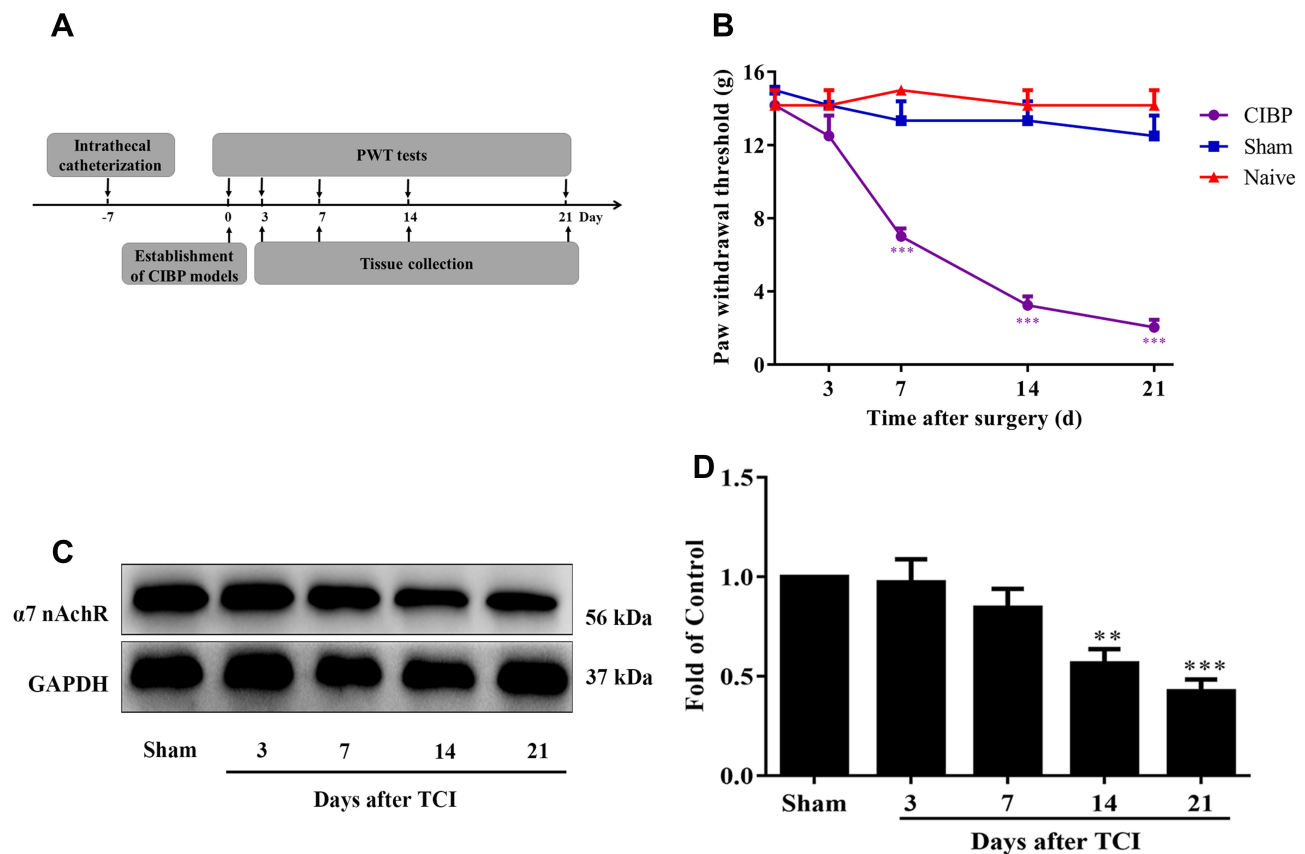


Figure 1 Spinal $\alpha 7$ -nAChRs was downregulated in CIBP rats. **(A)** Schematic diagram of the experimental procedure. **(B)** The ipsilateral PWTs in response to von Frey filaments decreased from day 7 to day 21 in CIBP rats. In contrast, the changes of PWTs in naive and sham rats were not observed in the 21 days (** $p < 0.001$ versus the naive group, $n = 6$ per group). **(C, D)** Western blot analysis indicated $\alpha 7$ -nAChRs protein level was gradually downregulated in CIBP rats (** $P < 0.01$, *** $P < 0.001$ versus the sham group, $n = 5$ per group).

Abbreviations: $\alpha 7$ -nAChR, alpha-7 nicotinic acetylcholine receptor; CIBP, cancer-induced bone pain; PWTs, paw withdrawal thresholds; TCI, tumor cells implantation.

$p = 0.0015$; $2.5 \text{ h}: F_{(4,17)} = 6.656$, $P = 0.0021$; $t = 3.725$, $p = 0.0168$) (Figure 3B). These results indicated that there were dose-dependent analgesic effects by pharmacological activation of $\alpha 7$ -nAChRs.

Positive Allosteric Modulator Intensified Analgesic Effects of $\alpha 7$ -nAChRs Agonist in CIBP Rats

Next, we verified whether $\alpha 7$ -nAChR positive allosteric modulator PNU-120,596 could intensify the analgesic effect of PNU-282,987. A relative lower dose of PNU-282,987 (0.1mg/kg) or $4 \mu\text{g}/10 \mu\text{L}$ PNU-120,596 was intrathecally administrated. When compared with rats given only lower dose of PNU-282,987, the analgesic effect of PNU-282,987 (i.t., 0.1mg/kg) on rats with pretreatment of $\alpha 7$ -nAChR positive allosteric modulator PNU-120,596, was enhanced at least at 0.5 h ($F_{(3,12)} = 7.73$, $p = 0.0039$; $t = 4.68$, $p = 0.0032$), 1.5 h ($F_{(3,12)} = 4.765$, $p = 0.0206$; $t = 3.772$, $p = 0.00160$) (Figure 3C). These results

showed that positive allosteric modulator PNU-120,596 could intensify the analgesic effect of PNU-282,987. Collectively, these results suggested positive allosteric modulator intensifies analgesic effects of $\alpha 7$ -nAChR agonist in CIBP rats.

Repeated Activation of $\alpha 7$ -nAChRs Reversed Downregulated Expression of $\alpha 7$ -nAChRs in Spinal Cord and Mechanical Allodynia State in CIBP Rats

The experimental design is shown in Figure 4A. We tested analgesic effect of chronic treatment 0.5mg/kg PNU-282,987 in CIBP rats. PNU-282,987 was administered once a day from day 14 to day 18 after tumor cells implantation. When compared with controlled rats, the PWTs were increased distinctly in CIBP rats treated with PNU-282,987 at day 14 ($F_{(3, 16)} = 89.33$, $p < 0.0001$; $t = 4.366$, $p = 0.0029$) day 15 ($F_{(3, 16)} = 78.96$, $P < 0.0001$; $t = 6.215$, $p < 0.0001$), day 16 ($F_{(3, 16)} = 50.75$, $P < 0.0001$; $t =$

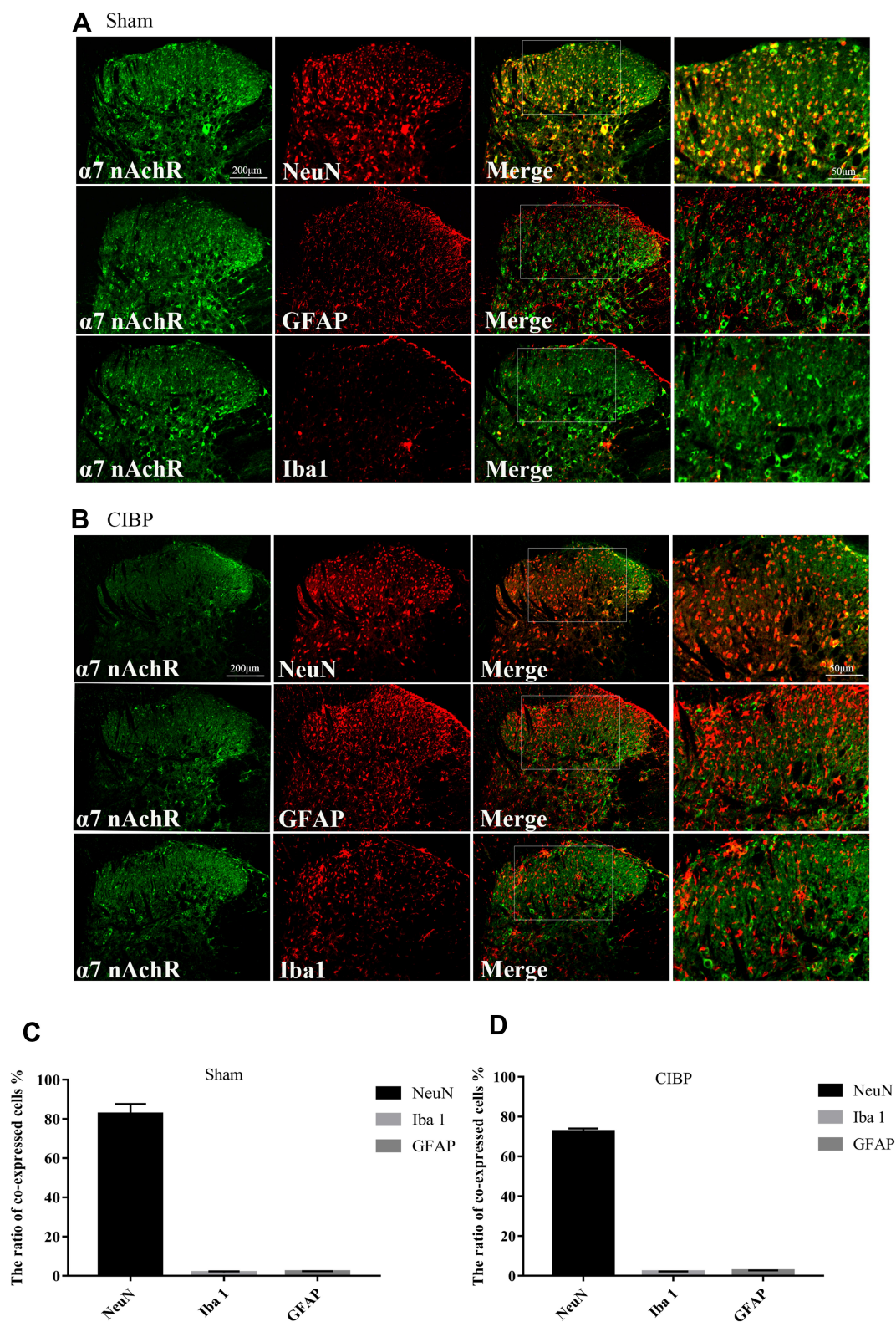
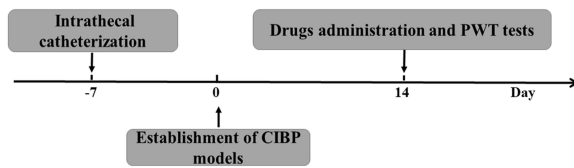


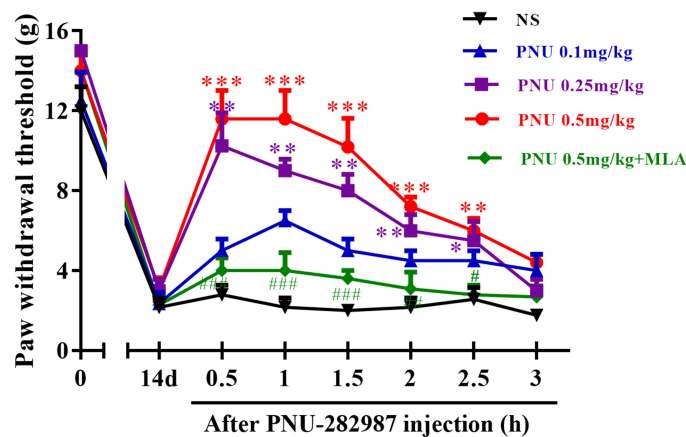
Figure 2 Expression and cellular localization of $\alpha 7$ -nAChRs in spinal cord dorsal horn. **(A, B)** Photomicrographs of $\alpha 7$ -nAChRs (green) double fluorescence labeling with neuronal marker NeuN (red), astrocyte marker GFAP (red), and microglial marker Iba1 (red) in the spinal cord at day 21 after TCI (n = 5 per group). Our results revealed that $\alpha 7$ -nAChRs were co-expressed predominantly with NeuN, only a few with GFAP and Iba1 in the spinal cord, both in CIBP and normal rats. In addition, immunofluorescence data showed that the immunoreactivity of $\alpha 7$ -nAChRs was decreased in the spinal cord dorsal horn on day 21 after TCI. **(C, D)** It was about 82.5% $\alpha 7$ -nAChRs in sham rats and 75.2% $\alpha 7$ -nAChRs in CIBP rats were expressed in neurons, and less than 5% of $\alpha 7$ -nAChRs were expressed on activated microglia and activated astrocytes.

Abbreviations: $\alpha 7$ -nAChR, alpha-7 nicotinic acetylcholine receptor; CIBP, cancer-induced bone pain; PWTs, paw withdrawal thresholds; TCI, tumor cells implantation.

A



B



C

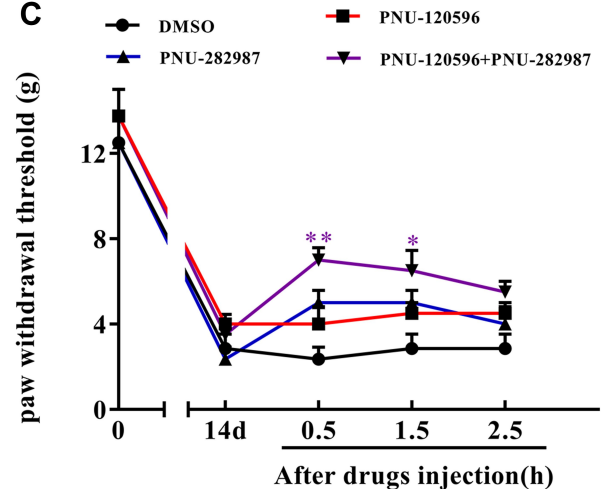


Figure 3 Pharmacologically activating $\alpha 7$ -nAChRs produce analgesic effect in CIBP rats. **(A)** Schematic diagram of the experimental procedure. **(B)** Single-dose PNU-282,987 (i.t., 0.5, 0.25 and 0.1 mg/kg) was given on day 21 after TCI. The PWTs were tested at 0.5h, 1h, 1.5h, 2h, 2.5h, and 3h after drug injection. PNU-282,987 at the dosage of 0.25 and 0.5 mg/kg dramatically reversed the PWTs in CIBP rats, lasting from 0.5h to 2.5 h. The MLA (i.t. 10 μ g/10 μ L) was administered 30 min before PNU-282,987 (i.t., 0.5mg/kg) injection. Results showed that MLA could block the analgesic effect of PNU-282,987. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the NS group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the group treated with PNU-282,987 0.5 mg/kg, $n = 5$ in 0.5mg/kg PNU group, MLA+0.5mg/kg PNU group; $n = 4$ in 0.25mg/kg PNU group, 0.1mg/kg PNU group and NS group; PNU=PNU-282,987). **(C)** PNU-282,987 (i.t., 0.1mg/kg) and PNU-120,596 (i.t., 4 μ g/10 μ L) on its own had no obvious analgesic effect in CIBP rats, respectively. However, the analgesic effect of PNU-282,987 (i.t., 0.1mg/kg) was intensified at least at 0.5h and 1.5h by 30min pretreatment of PNU-120,596 (i.t., 4 μ g/10 μ L). (* $P < 0.05$, ** $P < 0.01$ versus the DMSO group, $n = 4$ per group).

Abbreviations: $\alpha 7$ -nAChR, alpha-7 nicotinic acetylcholine receptor; CIBP, cancer-induced bone pain; PWTs, paw withdrawal thresholds; TCI, tumor cells implantation; MLA, methyllycaconitine; i.t., intrathecal catheterization.

6.335, $p < 0.0001$), day 17 ($F_{(3, 16)} = 42.48$, $p < 0.0001$; $t = 6.2$, $p < 0.0001$) and day 18 ($F_{(3, 16)} = 44.6$, $P < 0.0001$; $t = 5.511$, $p = 0.0002$) after tumor cells implantation (Figure 4B).

To explore whether repeated administration of PNU-282,987 played a role in analgesia by restoring the expression of $\alpha 7$ -nAChRs, the expression of $\alpha 7$ -nAChRs in the spinal cord of CIBP rats was tested by Western blot and immunofluorescence. All rats were conducted with PNU-282,987 (i.t., 0.5 mg/kg) or normal saline chronically from day 14 to day 18 after TCI. In CIBP rats, $\alpha 7$ -nAChRs protein level was significantly increased compared to rats treated with normal saline ($F_{(1,16)} = 45.33$, $P = 0.0090$; $t = 3.766$, $p = 0.0101$). Further, in sham rats, the protein expression of $\alpha 7$ -nAChRs also higher in PNU-282,987 treated rats than NS treated rats ($F_{(1,16)} = 45.33$, $P = 0.0090$; $t = 4.99$, $p = 0.0008$) (Figure 4C and D). In Figure 4E–I, immunofluorescence staining results

were consistent with the Western blot data, either in sham group ($F_{(1, 16)} = 45.59$, $p < 0.0001$; $t = 3.472$, $p = 0.0189$) or CIBP group ($F_{(1, 16)} = 45.59$, $p < 0.0001$; $t = 6.077$, $p < 0.00010$). Collectively, repeated activation of $\alpha 7$ -nAChRs could upregulate $\alpha 7$ -nAChRs expression and reverse mechanical allodynia in CIBP rats.

Repeated Activation of $\alpha 7$ -nAChRs Inhibited Spinal NF- κ B Signaling Pathway in CIBP Rats

To further explore the possible post-receptor mechanism of $\alpha 7$ -nAChRs, we tested the spinal expression of NF- κ B-p65 protein. Firstly, we tested the protein level of NF- κ B-p65 in CIBP rats. When compared with sham rats, the expression level of NF- κ B-p65 significantly increased in CIBP rats on day 21 after TCI ($t = 5$, $df = 8$, $p = 0.0011$) (Figure 5A

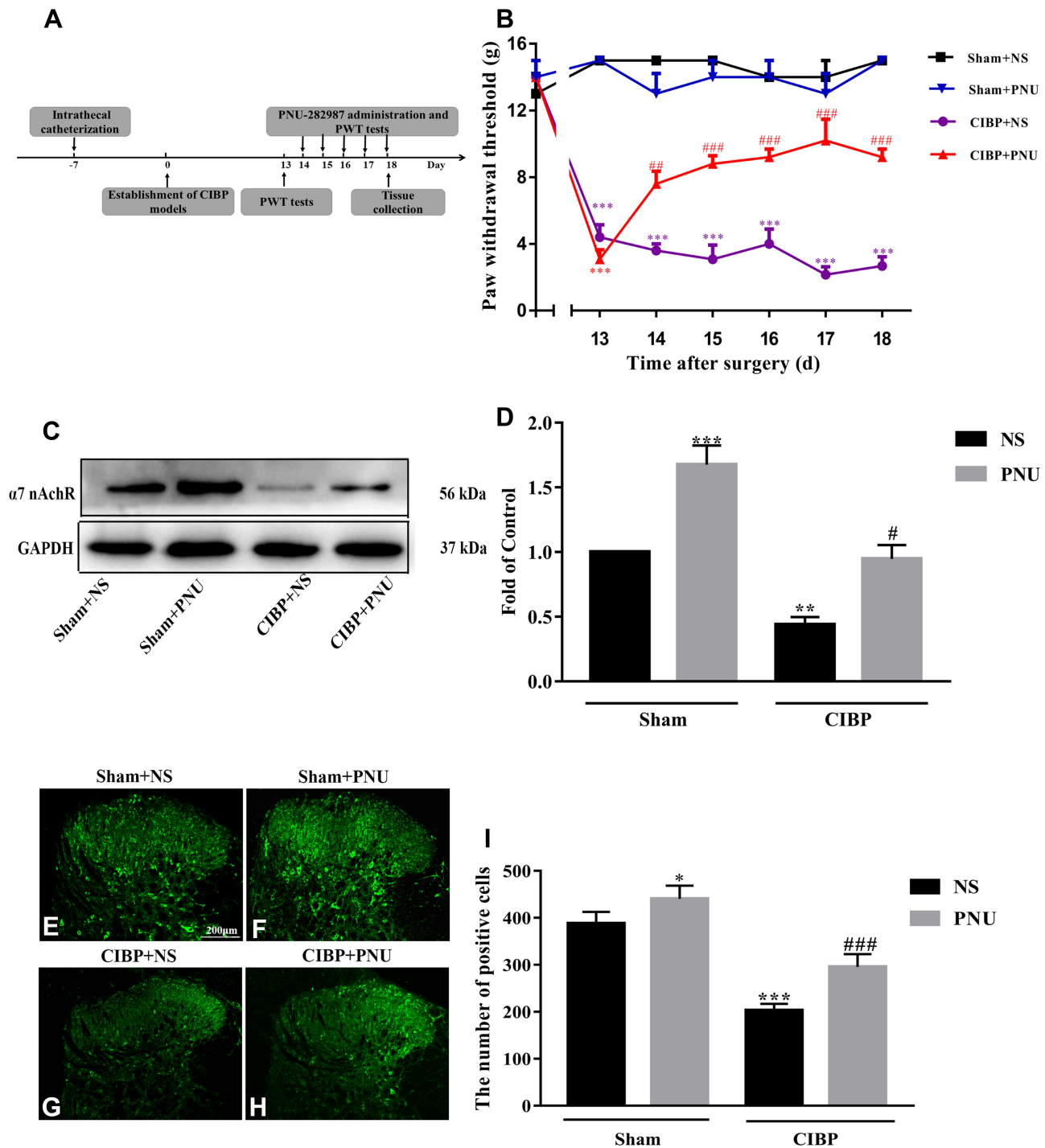


Figure 4 Repeat activating $\alpha 7$ -nAChRs reversed downregulation expression of $\alpha 7$ -nAChRs in spinal cord and mechanical allodynia state in CIBP rats. **(A)** Schematic diagram of the experimental procedure. **(B)** The dose of 0.5mg/kg PNU-282,987 was administered once daily from day 14 to day 18 after tumor cells implantation. The behavioral data was acquired at 2.0h after PNU-282,987 injection. End results showed that repeated injection of PNU-282,987 (0.5mg/kg, i.t.) alleviated the mechanical allodynia in CIBP rats. However, no obvious changes of PWTs were observed in CIBP + NS group (** $p < 0.01$, *** $p < 0.001$ versus the sham + NS group; ### $p < 0.01$, #### $p < 0.001$ versus the CIBP + NS group, $n=5$ per group; PNU=PNU-282,987). **(C, D)** Western blot data showed that PNU-282,987 upregulated the protein expression of $\alpha 7$ -nAChRs not only in CIBP rats but in sham rats (** $p < 0.01$, *** $p < 0.001$ versus the Sham+NS group, # $p < 0.05$ versus the CIBP+NS group, $n = 5$ per group; PNU=PNU-282,987). **(E–I)** Photomicrographs showed that PNU-282,987 reversed CIBP-induced downregulation of $\alpha 7$ -nAChRs immunoreactivity in the spinal cord in CIBP and normal rats. (* $P < 0.05$, *** $P < 0.001$ versus the Sham+NS group; #### $P < 0.001$ versus the CIBP+NS group, $n=5$ per group; PNU=PNU-282,987). PNU-282,987 (i.t., 0.5 mg/kg) or NS (10 μ L) was administrated once a day from day 14 to day 18 after TCI. All samples were harvested after PWT tests (PNU=PNU-282,987).

Abbreviations: $\alpha 7$ -nAChR, alpha-7 nicotinic acetylcholine receptor; CIBP, cancer-induced bone pain; PWTs, paw withdrawal thresholds; TCI, tumor cells implantation; NS, normal saline; i.t., intrathecal catheterization.

and B). Then, we evaluate its expression level after repeated PNU-282,987 (i.t. 0.5mg/kg) treatment for 5 days (from day 14 to day 18 after TCI). In CIBP rats when treated with PNU-282,987, NF- κ B-p65 protein level was obviously below the NS group ($F_{(1,16)} = 14.84$, $P=0.0014$; $t=5.786$, $p=0.002$). And in sham rats, the expression level of NF- κ B-p65 did not change whenever PNU-282,987 was given or not ($F_{(1,4)} = 22.54$, $P=0.0090$; $t=2.124$, $p=0.6053$) (Figure 5C and D). Therefore, it is likely that the spinal NF- κ B signaling pathway got involved in the post-receptor mechanism in the analgesic effect of PNU-282,987.

Discussion

The treatment of cancer-induced bone pain (CIBP) remains a major clinical challenge for its elusive mechanisms. The $\alpha 7$ -nAChRs are widely expressed in the pain transmission pathway and could be a potential non-opioid target for analgesia. Here in this study, by taking a CIBP model, we found spinal $\alpha 7$ -nAChRs contribute to the maintenance of CIBP.

The results revealed that spinal $\alpha 7$ -nAChRs mainly located in neurons and the expression of $\alpha 7$ -nAChR reduced at least from day 14 in a CIBP model. Previously,

investigators also found that the expression level of spinal $\alpha 7$ -nAChRs was downregulated in terms of both mRNA and protein in a rat oxaliplatin-induced neuropathic pain model.¹⁸ These findings indicate that the downregulation of $\alpha 7$ -nAChRs may be a vital contributor to the maintenance of chronic pain. Activation of $\alpha 7$ -nAChRs by its agonists has been reported to produce analgesic effects in several chronic pain models^{17-19,37-40} but lacks efficacy in some acute pain.⁴¹⁻⁴³ Here in the CIBP model, activation of $\alpha 7$ -nAChRs exerted analgesic effects in a dose-dependent manner and positive allosteric modulator PNU-120,596 intensified the effects of PNU-282,987. Thus, $\alpha 7$ -nAChRs could be a potential non-opioid target for CIBP. The reasons why $\alpha 7$ -nAChR agonist was valid in chronic pain, but invalid in some acute pain models have not to be revealed. One of the greatest possibilities could be its indirect action, by targeting NR2B-containing NMDA receptor, which is a key molecular of central sensitization^{44,45} and also largely involved in chronic pain state including CIBP.^{46,47} Activation of $\alpha 7$ -nAChRs inhibited phosphorylation of NR2B in the spinal dorsal horn,³⁴ and reduced the amplitude of NMDA mediated postsynaptic currents through

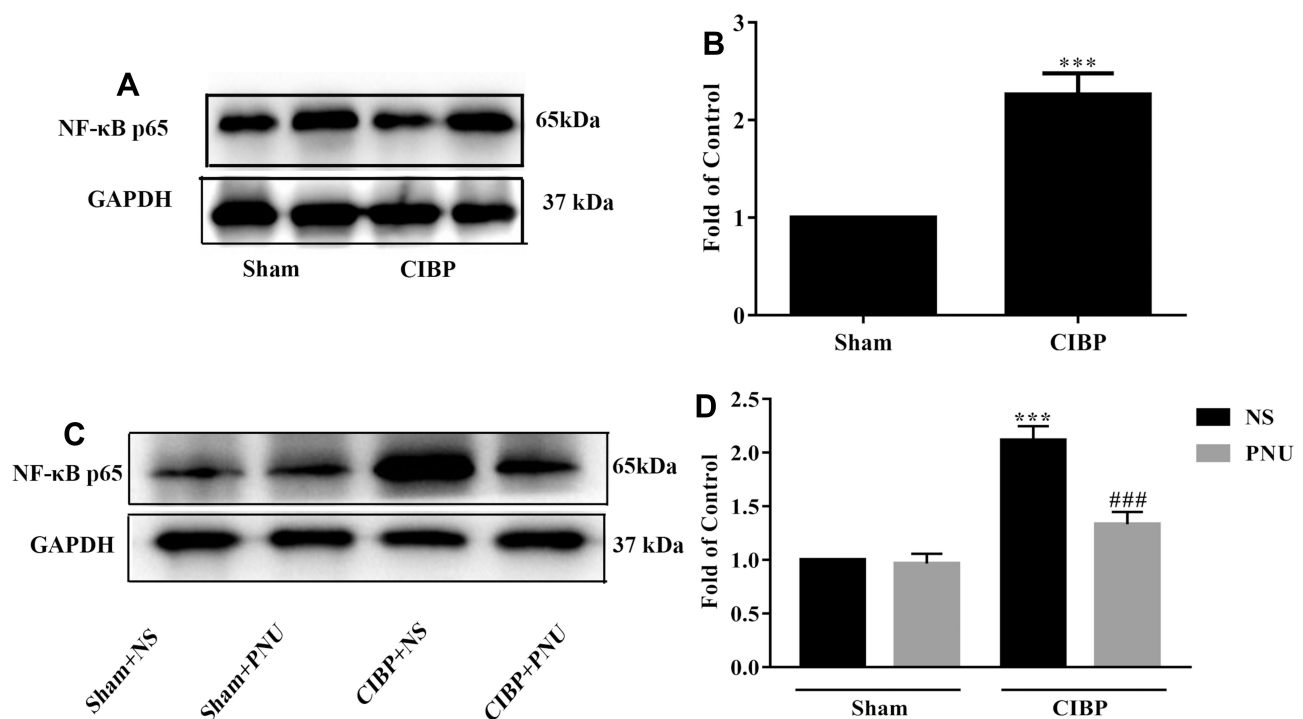


Figure 5 Repeat activating $\alpha 7$ -nAChRs inhibit spinal NF- κ B Signaling Pathway in CIBP rats. Western blot analysis indicated NF- κ B-p65 protein level increased on day 21 after TCI (***) $P < 0.01$ versus the sham group, $n = 5$ per group) (A, B). (C, D) Western blot analysis showed that after PNU-282,987 (i.t. 0.5mg/kg) treatment for 5 days (from day 14 to day 18 after TCI), the protein expression of NF- κ B-p65 was downregulated (***) $P < 0.001$ versus the Sham+NS group, ### $p < 0.001$ versus the CIBP+NS group, $n = 5$ per group; PNU=PNU-282,987).

Abbreviations: $\alpha 7$ -nAChR, alpha-7 nicotinic acetylcholine receptor; NF- κ B, nuclear factor kappa B; CIBP, cancer-induced bone pain; PWTs, paw withdrawal thresholds; TCI, tumor cells implantation; NS, normal saline; i.t., intrathecal catheterization.

a direct interaction with the NR2B subunit of the NMDAR.⁴⁸ As a result, the degree of central sensitization was lowered.

Another finding was that repeated activation of $\alpha 7$ -nAChRs could partially reverse mechanical allodynia state in CIBP rats. One reason could be restoring the expression of $\alpha 7$ -nAChRs. The protein level of $\alpha 7$ -nAChRs in CIBP rats was restored after repeated intrathecal treatment of PNU-282,987. Our findings were very consistent with some other studies. Christensen et al have discovered repeated administration of $\alpha 7$ -nAChR agonists could widely increase $\alpha 7$ -nAChRs expression in the juvenile rat brain.⁴⁹ Investigators demonstrated chronic PNU-282,987 treatment augments $\alpha 7$ -nAChRs expression in sciatic nerve, DRG and spinal cord of sham rats¹⁸ as well. The exact mechanisms for upregulation of $\alpha 7$ -nAChRs after repeated activation are not quite clear. Some researchers considered that, in the continuous presence of nAChR agonist, nAChRs might be locked in the membrane, the turnover and degradation pathway is reduced or prevented.⁵⁰ And some investigators thought it is about receptor trafficking, subunit maturation and assembly, changes in subunit stoichiometry and nAChR conformational changes.⁵¹ Another possible reason for chronic administration of $\alpha 7$ -nAChR agonist to alleviate mechanical allodynia in CIBP rats could be by inhibiting NF- κ B signaling pathway. The following relevant intracellular signaling pathways may be involved. Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) may participate in the prevention of NF- κ B/p65 activity after $\alpha 7$ -nAChRs activation.⁵² Besides this, JAK2/phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) pathway⁵³ and cyclooxygenase2 (COX2)/prostaglandin E2 (PGE2) pathway⁵⁴ are also involved. Specific as follows: the activation of $\alpha 7$ -nAChRs results in enhancement of JAK2-mediated tyrosine-phosphorylation of PI3K, AKT as well as activation of cAMP response element-binding protein (CREB).^{55–57} Besides these, $\alpha 7$ -nAChRs activation leads to stimulation of adenylate cyclase-1 (AC1) which then increases cAMP level, in turn, protein kinase A (PKA) is stimulated and CREB is activated ultimately.⁵⁸ Subsequently, activated CREB competes with NF- κ B for limited amounts of CREB binding protein (CBP)/p300 to inhibit NF- κ B activity.^{55–57} In this study, NF- κ B-p65 protein level was upregulated on day 21 after TCI; however, after chronic administration with PNU-282987, the protein expression of NF- κ B-p65 was downregulated, suggesting that activation of

$\alpha 7$ -nAChRs reverses mechanical allodynia in CIBP model by inhibition of NF- κ B signaling pathway.

In summary, we demonstrated that spinal $\alpha 7$ -nAChRs contribute to the maintenance of CIBP, activation of $\alpha 7$ -nAChRs produced a powerful analgesic effect. A positive allosteric modulator could intensify its effects. And recovery of $\alpha 7$ -nAChRs expression attenuated CIBP-induced mechanical allodynia, at least partly, by inhibiting the NF- κ B signaling pathway in this study. Thus, $\alpha 7$ -nAChRs could be a potential non-opioid pharmacological target for CIBP.

Conclusion

Our research demonstrated that spinal $\alpha 7$ -nAChRs were downregulated which mediated pain hypersensitivity in bone cancer pain, and restoring diminished $\alpha 7$ -nAChRs attenuates CIBP-induced mechanical allodynia, at least partly, by inhibiting the NF- κ B signaling pathway in this study. Therefore, this new knowledge indicated that spinal $\alpha 7$ -nAChRs may become a valuable target for the therapy of CIBP.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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