C/EBPβ Participates in Nerve Trauma-Induced TLR7 Upregulation in Primary Sensory Neurons

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Research Article

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Abstract

Nerve trauma-induced toll-like receptor 7 (TLR7) expression level increase in primary sensory neurons in the damaged dorsal root ganglion (DRG) avails to neuropathic pain, but the reason is still unknown. In the current study, we showed that unilateral lumbar 4 (L4) spinal nerve ligation (SNL) upregulated CCAAT/enhancer-binding protein-β (C/EBPβ) expression in ipsilateral L4 DRG. Preventing this elevation attenuated the SNL-induced upregulation of TLR7 in the ipsilateral L4 DRG and inhibited cold/thermal hyperalgesia and mechanical allodynia. Mimicking nerve trauma-induced C/EBPβ upregulation generated an elevated level of TLR7 in injected DRG, augmented responses to cold/thermal/mechanical stimuli while causing ipsilateral spontaneous pain with no SNL. Mechanistically, SNL upregulated binding of increased C/EBPβ to *Tlr7* promoter in ipsilateral L4 DRG. Accorded that C/EBPβ could trigger the activation of *Tlr7* promoter and co-expressed with *Tlr7* mRNA in individual DRG neurons, our findings strongly suggest the role of C/EBPβ in nerve trauma-mediated TLR7 upregulation in damaged primary sensory neurons.

Introduction

Neuropathic pain refers to chronic pain resulting from central or peripheral nervous system (CNS, PNS) disease or injury, which poses a massive challenge to physicians due to poor response to conventional pain treatments. About 3.3–17.9% of the United States and European population suffers from neuropathic pain [1]. It is identified based on persistent pain of spontaneous onset, cold/thermal hyperalgesia, spasmodic burning pain, and allodynia. Currently, some treatments are available to treat neuropathic pain, like non-steroidal anti-inflammatory drugs (NSAIDs), opioids, antidepressants, and anti-convulsants [1–3]. Nonetheless, many cases do not achieve sufficient pain relief using the existing therapeutics since they mostly lack specificity to neuropathic pain [1,3]. Thus, discerning the underlying mechanisms of neuropathic pain could provide new and more efficient therapeutic strategies for treating neuropathic pain.

After peripheral nerve injury, hyperexcitability and abnormal deranged firing in primary sensory neurons in dorsal root ganglion (DRG), as well as neuroma in the injury position, have been suggested to promote neuropathic pain in the PNS [2,4]. DRG neurons' aberrant spontaneous activity together with consequently heightened neurotransmitter release from their primary afferent may be associated with maladaptive changes, just as enzyme and receptor translation, gene transcription, together with voltage- and ligand-dependent ion channels in DRG [5–8]. In mammals, Toll-like receptors (TLRs) play essential roles in inducing the innate immune responses to molecular patterns related to a pathogen. Previous findings confirm that the TLRs family contains 13 proteins (TLR1–13) in mammals. Toll-like receptors 7 (TLR7) recognize single-stranded nucleic acids from RNA viruses and signals *via* myeloid differentiation factor 88 (MyD88) the production of cytokines and chemokines to fight against pathogenic infection [9].

Cumulative evidence suggests that TLR7 is highly implicated in abnormal pain hypersensitivity and itch. Approximately 33% of DRG neurons are positive for TLR7, about 55% are small [10]. TLR7 is co-expressed

with TRPV1, GRP, TRPA1, and MrgprA3 in DRG neurons [11,12]. Natural and synthetic TLR7 ligands are potent adjuvant for rapid action potential and inward current of nociceptive neurons in DRG [11]. In our previous study, trauma to the PNS resulting from spinal nerve ligation (SNL) significantly increased TLR7 expression in damaged DRG neurons at mRNA and protein levels [10]. Blocking this increase by virus-mediated knockdown of TLR7 in DRG could alleviate the cold-/thermal-related nociceptive hypersensitiveness and SNL-mediated mechanical allodynia in mice, regardless of gender. Mimicking this increase by overexpression of TLR7 in naïve mice DRG could elicit neuropathic pain symptoms, including hypersensitivity to mechanical, heat, and cold [10]. Mechanistically, elevated TLR7 promoted the p65 (NF-κB transcription factor (TF) family members) nuclear import and phosphorylation in the injured DRG neurons [10]. In primary sensory neurons, peripheral nerve trauma-induced DRG TLR7 increase mainly induced neuropathic pain through the activation of the NF-κB pathway [10]. Nevertheless, how peripheral nerve trauma transcriptionally activates TLR7in injured DRG is still elusive.

CCAAT/enhancer-binding protein-β (C/EBPβ) belongs to the TF family, which has a high conservation degree of the DNA binding-related basic-leucine zipper domain [13]. Our study adopted the JASPAR database to identify consensus-binding motifs (-138 to –128 and –56 to –46) of C/EBPβ in the *Tlr7* promoter gene. In the present study, we first examined whether the level of C/EBPβ expression was increased in injured DRG after SNL. Our study observed whether blocking the increased DRG C/EBPβ alleviated SNL-induced elevation of TLR7 in injured DRG and nociceptive hypersensitivity. Effects of mimicking SNL-induced DRG C/EBPβ increase on DRG TLR7 expression and basic nociceptive threshold were also examined. Finally, whether C/EBPβ directly bonded to and triggered the *Tlr7* promoter in injured DRG after SNL was investigated.

Materials And Methods

Animal preparation

The present work adopted 7–8-week-old CD-1 male adult mice provided by Charles River Laboratories (Beijing, China). All mice were raised within a housing room under 12-h/12-h light/dark cycle conditions and were allowed to eat food and drink water freely. Our study protocols were approved by the Institutional Animal Care and Use Committee at Zhengzhou University, China. The animals were adaptively fed for 2–3 days prior to behavioral tests. The treated groups were blinded for experimenters.

Neuropathic pain models

Our study constructed a mouse model of neuropathic pain by lumbar 4 (L4)SNL in line with the previous description [14–17]. Briefly, under 1.5 – 2.5% sevoflurane inhaled anesthesia and on a warm surface, an incision was made in the midline of lower back skin on L5–L2 vertebrae. The left L4 spinal nerve was exposed carefully by blunt dissection and removal of the overlying transverse vertebral process, followed by tight ligation using the 6–0 silk suture and transection at the distal ligation site under a surgical microscope. Identical procedures were carried out in sham groups with an exception for ligation and transection.

Behavioral tests

All behavioral testing was accomplished by one investigator who was blinded with the group. Five behavioral tests were carried out as described previously, including mechanical, thermal, and cold tests, in addition to locomotor activity and conditional place preference (CPP) tests [18–21]. Each evoked behavioral test was committed at 30 min intervals. The animals were placed inside individual Plexiglas chambers onto the rising mesh screen to assess mechanical allodynia. After that, 0.4 and 0.07 g von Frey filaments (Stoelting Co.) after calibration were used to the sciatic territory of the plantar hind paw ten times at the intervals of 5 min. The rapid paw withdrawal response number was recorded in 10 tests as the paw withdrawal frequency [response frequency =(paw withdrawal number/10 test) × 100%]. Thermal allodynia was evaluated by calculating the latency to withdrawal from a radiant heat source, and the animals were put into individual Plexiglas chambers onto the surface of the glass plate. We applied the light of variable intensity penetrating from Model 336 Analgesia Meter lightbox (IITC Inc. Life Science Instruments. Woodland Hills, CA) onto the glass plate to the middle of the hind paw plantar surface. An automatic light beam shut was completed in the case of foot withdrawal by the mouse. Then, the duration from light beam start to shut was recorded as paw withdrawal latency. We conducted this test 5 times for every animal at intervals of 5 min. A cut-off time of 20 s was applied to avoid tissue damage. The mice were also placed in the individual Plexiglas chambers under 0°C onto a cold aluminum plate to evaluate cold hyperalgesia. Then, the period from placing the animal onto the plate to a quick jump with/without paw flinching/shanking on the ipsilateral side was recorded as paw withdrawal latency. This test was conducted thrice for each animal at intervals of 10 min. For avoiding damage to surrounding tissues, 20 s was set as the threshold time. The CPP device comprising two different chambers connected to the inside gate (MED Associates In., St. Albans, VT) was employed for the CPP test. One chamber was composed of black/white horizontal stripes wall and a rough floor, while the other had black/white vertical stripes and a smooth floor. The photo beam detector was installed against the top of chamber walls to monitor mouse motion, and software was utilized to record time spent in every chamber automatically. Firstly, the mice could enter the two chambers for 30 min.

After this pre-conditioning, the baseline time spent within every chamber in a 15-min period was recorded. The mice spending lower than 180 s or over 720 s in either chamber were eliminated for subsequent tests. Within the next three days, the inside gate was closed, and the conditioning protocols were executed. In the morning on day 1, the mice were pooled with intrathecally injected 5 μL saline in one conditioning chamber. After six hours in the afternoon, the mice were pooled with intrathecal injection of 0.8% lidocaine (dilution in 5 μL of saline) in the other conditioning chamber. The lidocaine and saline injection procedures were changed daily. On day 4 (examination day), the mice were allowed to enter the two chambers. The duration spent in an individual chamber within 15 min was recorded. The difference score was the difference between the basal and test durations spent within the lidocaine chamber. Prior to euthanasia, locomotor activity tests were conducted, including placing, righting, and grasping reflexes. Placing reflex was measured when the dorsal surface of the hind-paws came in contact with the table edge, with the hind-limb position slightly inferior to forelimbs. The investigator recorded the reflexive or non-reflexive placement of hind-paws onto the table surface. Righting reflex was performed with the

mouse back onto the flat surface. The investigator recorded the immediate returning of mice to the normal upright position. A grasping reflex was performed with the animals being placed onto the wire grid. The investigator recorded the grasping of wire-on-contact by the hind-paws. All the trials are repeated five times with a 10 min intermission. The scores of each reflex were determined based on counts of each normal reflex.

Dorsal root ganglion microinjection

DRG microinjection was conducted according to the previous description after slight modification [22,23]. In brief, 2% sevoflurane was injected into each mouse for anesthesia, then an incision was made in the middle of the lower back. Articular processes in L3/L4 vertebrae were further dissected with caution and eliminated using small rongeurs. Following DRG exposure, the siRNA (1 μL, 20 mM) or viral (1 μL, 2×10^{14} TU/mL) solution were injected in unilateral L3/L4 DRG using a Hamilton syringe connected to a glass micropipette. After each DRG injection, the glass micropipette was retained for 10 min before removing it. Sterile saline was used to inundate the surgical field, followed by surgical incision closure using metal wound clips. Animals that exhibited aberrant locomotor activities were eliminated.

Cell culture and transfection

DRG neurons and HEK-293T cells were cultured according to previously described methods [24,25]. Briefly, HEK-293T cells were cultivated in the DMEM/high glucose (HG) medium (Gibco ThermoFisher Scientific, Waltham, MA, USA) that contained 1% antibiotics and 10% fetal bovine serum (FBS) (Gibco ThermoFisher Scientific, Waltham, MA, USA). To prepare the primary DRG neuron culture, 4% sevoflurane was first used to euthanize CD1 male mice (4-week-old), then bilateral DRGs were collected in a cold neurobasal medium (Gibco ThermoFisher Scientific, Waltham, MA, USA) that contained 10% FBS and 1% antibiotics. The tissues were incubated with Hanks' balanced salt solution (HBSS) (Gibco ThermoFisher Scientific, Waltham, MA, USA), including 1 mg/mL collagenase type I and 5 mg/mL dispase for a 2-h period at 37°C, followed by 10 min of digestion using 0.25% trypsin (Cellgro) at 37°C and then using 0.25% trypsin inhibitor (Sigma). After trituration and centrifugation, the dissociated neurons were resuspended in a neurobasal defined medium containing 2% B27 supplement (Invitrogen), which were later placed into the 6-well plate containing poly-D-lysine (50 μg/mL, Sigma). The neurons were further cultivated at 37°C and in 5% CO_2. After 24 h, 2 μL of AAV5 (titer $\geq 1 \times 10^{13}$/μL) or 100 nM siRNA (diluted with Lipofectamine 2000) was supplemented to 2-ml well each. After three days, the culture neurons were harvested for Western blot.

Western blot (WB) analysis

Four unilateral L4 DRGs collected in 4 animals were mixed to prepare the homogenate to achieve adequate protein for analysis. After tissue homogenization, the cultivated cells were exposed to ultrasonication in a cool lysis buffer. The homogenates were centrifuged at 4°C for 10 min at 10,000 g. Later, supernatants were obtained to analyze the cytosolic proteins and the centrifuged pellets to analyze

the nuclear proteins. This study utilized the BCA protein assay kit ((Pierce, Rockford, USA)) to determine protein content. After heating for 6 min at 95°C, equivalent quantities of protein (30 mg/Lane) were loaded on a 5% stacking/7.5% resolving SDS-PAGE gel, followed by transfer onto the PVDF membranes (250 mA, 2 h). The membranes were first blocked for 1 h with 5% skimmed milk in Tris-buffered supplemented with 0.1% Tween-20 at 37°C. Later, primary antibodies were used to incubate membranes overnight at 4°C, including against rabbit anti-C/EBPβ; (1:1,000, ab32358, Abcam, USA), rabbit anti-TLR7 (1:500; ab113524, Abcam, USA), rabbit anti-GAPDH (1:2,000; cat88845, CST, USA), rabbit anti-H3 (1:1,000; PA5–16183, Invitrogen, USA) , mouse anti-GFAP (1:1,000; ab7260, Abcam, USA), rabbit anti-phosphorylated ERK (p-ERK; 1:1,000; ab207470, Abcam, USA), rabbit anti-total ERK (1:1000; ab109282, Abcam, USA). Membranes were further incubated with HRP-labeled anti-rabbit or anti-mouse secondary antibody (1:2,000; Invitrogen, USA) and visualized using RapidStep™ ECL Reagent (345818, Merck Millipore, USA) and exposure using ChemiDoc Systems with ImagePro Lab software (Bio-Rad, USA). ImagePro software was used for image acquisition and densitometric analysis of the bands. GAPDH was used to normalize against all cytosol proteins, whereas histone H3 was used to normalize against all nucleus proteins.

Quantitative RT-PCR (qRT-PCR) assay

Total RNAs were extracted from DRGs using the RNA-Solv Reagent (Omega, BioTeK, GA) following the manufacturer's instructions. Reverse transcription was organized with 2 μg of RNA using ReverTra Ace (TOYOBO, Osaka, Japan) and Oligo(dT) (TaKaRa, Japan). Then, the prepared cDNA was run in the 20-μL reaction system containing Advanced Universal SYBR Green Supermix (10 μL, Bio-Rad Laboratories, USA) and forward and reverse primers (Table 1, 250 nM each) in triplicate. The ViiA 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA) was employed to carry out qRT-PCR procedures. The cycling parameters were as follows: the mixture was incubated at 95°C for 5 min, followed by 45 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 10 s. The relative mRNA transcript level was determined by the ΔΔCt method.

Our study conducted single-cell RT-PCR assays according to the previous description [26,27]. In brief, the fresh DRG neurons from 3–4 weeks old mice were prepared. Three hours after plating, the size of the living single-DRG-neuron was calculated using an inverted microscope, and neurons were harvested using the glass microneedle. The collected neurons were placed in individual PCR tubes using cell lysis buffer (10 μL, Thermo Fisher Scientific). The cells were incubated for 10 min on ice and centrifuged at 4°C and 10,000 g for 5 min to collect the supernatants. RT-PCR was conducted according to specific protocols using the single-cell RT-PCR assay kit (Thermo Fisher Scientific, Waltham, USA). The primers for single-cell RT-PCR are listed in Table 1.

Plasmid construction and virus production

The pcDNA3.1(-) plasmid harboring full-length mouse C/EBPβ was offered by Dr. Xi Li (Fudan University, Shanghai, China) [28]. After XbaI/BamHI digested the plasmid, C/EBPβ cDNA was purified and ligated into pro-viral plasmids (UNC Vector Core, CA). The C/EBPβ-expression vector was obtained,

controlled by the cytomegalovirus promoter. DNA sequencing was performed to verify the recombinant clones. Our study adopted the AAV-DJ Helper Free Packaging System (Cell Biolabs, Inc., CA) to prepare AAV-DJ viral particles and purified using the AAV pro Purification Kit (Takara, Mountain View, CA).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed based on the EZ-ChIP kit (Millipore, Germany), following the manufacturer's instructions with minor modifications. Five unilateral L4 DRG from 5 SNL or sham mice were harvested and put together. After the homogenization, 1% formaldehyde was adopted for 10 min of homogenate cross-linking at 37°C, then 0.125 M glycine solution was added to quench reaction under ambient for 5 min. Further, the pellet was obtained by cold centrifugation at 1000 rpm for 5 min. After washing, the obtained pellet was prepared into suspension with protease inhibitor cocktail-containing lysis buffer that included 0.1% SDS, 1% NP-40, and 1% sodium deoxycholate. The chromatin was sheared by sonication of the suspension to generate the DNA fragments (0.2–1 kb). Subsequently, protein G agarose was added to pre-clear the samples under 4°C for 2 h, and immunoprecipitated using normal rabbit serum (2 μg) or rabbit anti-C/EBPβ antibody (2 μg, Abcam, USA) at 4°C overnight, with 10% sample being the positive control for immunoprecipitation. PCR or qRT-PCR assays were conducted to purify and identify the DNA fragments. Table 1 presents all the primers used.

Dual-Luciferase assay

The 752-bp fragment (-689 to +62) of the *Tlr7* gene promoter region (including 2 C/EBPβ-binding motifs) was amplified based on the genomic DNA (gDNA) using PCR to manufacture *Tlr7* gene reporter plasmid. Table 1 lists the primers used. PCR products were inserted into the firefly luciferase reporter gene-containing pGL3-Basic vector by adopting Hind-III and Kpn-I restriction sites. DNA sequencing was performed later to confirm recombinant plasmid sequences.

HEK-293T cells were cultured as described above. After incubation (24 h), Lipofectamine 2000 (Invitrogen, USA) was used to transfect the cells using URL-TK plasmid (40 ng, the Revilla luciferase reporter gene-containing control) with/without pGL3-Basic vectors (1 mg) in line with specific protocols. The cells were harvested in a passive lysis buffer 48 h after transfection. In triplicate, the luciferase activity was quantified in the supernatant using the Dual-Luciferase Reporter Assay System (Invitrogen, USA). The relative reporter activity was calculated when the firefly luciferase activity was normalized to refill activity.

Statistical analysis

Animals were randomly allocated to different treatment groups. The data were expressed as means ±S.E.M and analyzed by one- or two-way ANOVA (with a column factor treatment and row factor time') and the paired, two-tailed Student's t-test. The *post hoc* Tukey test was applied to compare means between two groups for statistically different results from ANOVA analysis. $P<0.05$ was considered

statistically significant. GraphPad Prism (GraphPad Software 8.0, USA) was used for the statistical analysis.

Results

C/EBPβ expression is increased in injured DRG after SNL

Before we explored the participation of C/EBPβ in SNL-induced TLR7 transcriptional activity in injured DRG, the change in C/EBPβ level was examined in two regions associated with pain, including DRG, and spinal cord, following SNL or sham surgery. Rather than sham surgery, SNL induced the constantly elevated C/EBPβ protein expression in ipsilateral L4 (damaged) DRG. On day three post-SNL, the expression of C/EBPβ protein increased 5.41–fold, while on days 7 and 14, it increased by 6.34 and 5.95 folds, respectively, compared with the matched value following sham surgery (Fig. 1a). Both SNL and sham surgery did not change the baseline C/EBPβ protein levels in ipsilateral L3 (undamaged) DRG, ipsilateral L4 spinal cord, and contralateral L4 DRG (Fig. 1b). SNL-induced C/EBPβ increase in DRG indicates an underlying role of C/EBPβ in neuropathic pain.

Blocking the increased C/EBPβ in injured DRG attenuates SNL-induced neuropathic pain

In order to study whether C/EBPβ up-regulation in damaged DRG involved neuropathic pain generation, this study analyzed how C/EBPβ knockdown in the damaged DRG affected SNL-mediated pain hypersensitivity induction. Microinjection of specific C/EBPβ small interfering RNA (siRNA) in ipsilateral L4 DRG changed SNL-mediated nociceptive hypersensitivity development. As expected, at seven days post-SNL, the expression of C/EBPβ in ipsilateral L4 DRG elevated in animals with microinjection of negative control siRNA (NC-siRNA), compared with sham surgery group administered with microinjection of negative control siRNA (Fig. 2a). SNL mice given C/EBPβ siRNA microinjection did not show elevated C/EBPβ expression (Fig. 2a). There was no significant alteration in the basal C/EBPβ protein expression in ipsilateral L4 DRG from mice given microinjection of C/EBPβ siRNA (Fig. 2a). Based on these findings, microinjection with C/EBPβ siRNA ahead of time prevented the SNL-mediated C/EBPβ up-regulation in damaged DRG.

Consistent with previous studies [15,16,19], SNL produced permanent ipsilateral cold/thermal hyperalgesia and mechanical allodynia in mice given microinjection of NC-siRNA (Fig. 2b-e). Relative to baseline data before injection, at 3, 5, and 7 days after SNL, the paw withdrawal frequencies of ipsilateral hind-paw in response to mechanical stimuli elevated significantly (Fig. 2b-c). Besides, for ipsilateral hind-paw, its paw withdrawal latency upon cold and thermal stimuli decreased dramatically at 3, 5, and 7 days after SNL compared to before injection (Fig. 2d-e). In addition, C/EBPβsiRNA injection ahead of time had no difference to ipsilateral paw responses in the presence of thermal/cold/mechanical stimuli in mice receiving sham surgery (Fig. 2b-e) and reduced the SNL-mediated cold/thermal hyperalgesia and mechanical allodynia (Fig. 2b-e). Expectedly, SNL-mediated cold/thermal hyperalgesia and mechanical allodynia were still observed in the ipsilateral side of negative control siRNA-microinjected mice in the

observation process (Fig. 2b-e). Besides, siRNA injection ahead of time had no difference to contralateral baseline paw responses of SNL and sham mice (Fig. 2f-h) or the locomotor activity (Table 2).

This study further analyzed the influence of C/EBPβ siRNA pre-microinjection on the SNL-mediated central sensitization of dorsal horn, revealed by the increased phosphorylation of glial fibrillary acidic protein (GFAP, the astrocyte hyperactivation marker) and extracellular signal-regulated kinase 1/2 (p-ERK1/2, the neuronal hyperactivation marker) in the dorsal horn [14,25]. Conforming to research based on neuropathic pain model induced by chronic constriction injury or spinal nerve injury [14,25], at seven days post-SNL, the protein expression of the phosphorylation of ERK1/2 (rather than total ERK1/2) and GFAP elevated significantly in ipsilateral L4 dorsal horn of SNL mice given microinjection of NC-siRNA, compared to the negative control siRNA-microinjected sham mice (Fig. 3). Such changes disappeared in SNL mice with microinjection of C/EBPβ siRNA (Fig. 3). Neither C/EBPβ siRNA nor negative control siRNA changed the baseline phosphorylation levels of GFAP, total ERK1/2, and ERK1/2 in the dorsal horn of mice receiving sham surgery (Fig. 3). Overall, based on this study, C/EBPβ up-regulation in the damaged DRG played an essential role in the central sensitization of the dorsal horn and pain hypersensitivity induced by SNL.

Mimicking the SNL-induced increase in C/EBPβ in injured DRG leads to neuropathic pain symptoms

This study also investigated the sufficiency of C/EBPβ up-regulation in DRG for neuropathic pain mediated by SNL. In this regard, AAV5 expressing full-length C/EBPβ (AAV5-C/EBPβ) was micro-injected in unilateral L3/L4 DRGs into naïve male adult mice, while AAV5 that expressed green fluorescent protein (AAV5-GFP) served as the control. C/EBPβ protein expression substantially increased in microinjected DRGs in week eight following AAV5-C/EBPβ microinjection, rather than AAV5–GFP (Fig. 4a). Upon mechanical stimulation, mice given microinjection of AAV5-C/EBPβ (rather than AAV5–GFP) showed significantly increased paw withdrawal frequency (Fig. 4b-c) along with ipsilateral cold allodynia and thermal hyperalgesia, indicated by the decreased paw withdrawal latency upon cold and thermal stimuli, respectively (Fig. 4d-e). The nociceptive hypersensitivity appeared in the 4^{th} week, which lasted for eight or more weeks (Fig. 4b-e), conforming to the 3–4-week lag period of AAV5 level that persisted for three or more months [14,25]. AAV5-GFP and AAV5-C/EBPβ did not change the contralateral baseline paw responses upon cold/thermal/mechanical stimuli (Fig. 4b-e) or the locomotor activity (Table 2).

Besides, to test the stimuli-induced nociceptive hypersensitivity, the CPP paradigm was adopted to test the effect of mimicking SNL-mediated C/EBPβ up-regulation in DRG on activating the spontaneous persistent nociceptive responses. The mice microinjected with AAV5-C/EBPβ exhibited a significant preference towards the lidocaine-paired chamber (Fig. 4f-g), demonstrating that the spontaneous pain response was independent of stimuli. The mice microinjected with AAV5-GFP showed no distinct preference for lidocaine-or saline-paired chamber (Fig. 4f-g), indicating no marked spontaneous pain responses. These data suggest that increased C/EBPβ in DRG produces typical neuropathic pain symptoms, as both spontaneous and evoked nociceptive hypersensitivity.

The above results obtained from behavioral tests were evidenced by central sensitization of the spinal cord dorsal horn. In week eight post-AAV5-C/EBPβ microinjection, the GFAP and p-ERK1/2 levels elevated remarkably in the ipsilateral L3/4 spinal cord dorsal horn in comparison with post-AAV5-GFP microinjection (Fig. 5).

C/EBPβ transcriptionally activates the Tlr7 gene after SNL in injured DRG

Our study also examined the role of SNL-mediated C/EBPβ up-regulation in activating the *Tlr7* gene transcriptionally in the damaged DRG. At seven days post-SNL, pre-microinjection of C/EBPβ siRNA in DRGs, rather than NC-siRNA, suppressed the SNL-mediated TLR7 protein increase in ipsilateral L4 DRG (Fig. 6a,). At seven days after sham surgery, pre-microinjection of C/EBPβsiRNA did not involve the basal TLR7 protein expression in ipsilateral L4 DRG (Fig. 6a). Consistently, in week 8, AAV5-C/EBPβ microinjection, rather than AAV5-GFP, in unilateral L3/4 DRGs led to TLR7 upregulation in microinjected DRG (Fig. 6b). For a better understanding of how C/EBPβ affected TLR7 level in DRG neurons, full-length C/EBPβ through AAV5-C/EBPβ was transduced into cultured DRG neurons to overexpress C/EBPβ. C/EBPβ overexpression significantly increased the TLR7 level (Fig. 6c). Such elevation disappeared in the cultured neurons after co-transfection with C/EBPβ-specific siRNA and AAV5-C/EBPβ (rather than NC-siRNA) (Fig. 6c), revealing TLR7 upregulation was specific in response to C/EBPβ. In addition, transfection ofC/EBPβ siRNA alone also reduced the basal TLR7 expression (Fig. 6c). Thus, our data imply that C/EBPβ may directly regulate TLR7 in DRG neurons under neuropathic pain conditions.

To better confirm whether C/EBPβ directly affected *Tlr7* gene expression, ChIP assays were carried out. At seven days after sham surgery, a *Tlr7* promoter fragment, including C/EBPβ's binding motifs, was amplified from C/EBPβ antibody-immunoprecipitated complex from ipsilateral L4 DRGs in nuclear fractions (Fig. 7a). However, amplification using normal serum could not be conducted (Fig. 7a), indicating that the binding of C/EBPβ to *Tlr7* promoter was specific. At seven days post-SNL, C/EBPβ had markedly elevated binding activity to the *Tlr7* promoter in the damaged DRG, evidenced by the 13 times increased band density in ipsilateral L4 DRGs of SNL mice in comparison with sham mice (Fig. 7a). Based on these results, binding at seven days post-SNL was possibly ascribed to the elevated C/EBPβ protein expression in ipsilateral L4 DRG (Fig. 1a). Moreover, co-transfection with the full-length C/EBPβ vector, rather than the control *Gfp* vector, evidently enhanced the *Tlr7* gene promoter's transcription activity, as evidenced by luciferase assay results using HEK-293T cells (Fig. 7b). Additionally, C/EBPβ mRNA was co-expressed with TLR7 mRNA in all DRG neurons of all sizes (small, moderate, large), as evidenced by single-cell RT-PCR assay (Fig. 7c). These data suggest that the SNL-mediated C/EBPβ up-regulation promotes the *Tlr7* promoter binding activity, leading to the increases in TLR7 mRNA transcription and translation in injured DRG.

Discussion

Peripheral neuropathic pain primarily resulted from peripheral nerve injury leading to DRG neurons with axotomized (non-conducting) and uninterrupted fibers, which may conduct in the corresponding receptive

fields. In human beings, neuropathic pain has the feature of spontaneous pain or evoked pain caused by hypersensitivity to the normally non-painful (allodynia) or painful (hyperalgesia) stimuli. Investigating how peripheral nerve trauma produces hypersensitivity may provide a new route to understand, prevent, and treat this disturbance. Our previous study showed that SNL induced upregulated expression of TLR7 in the injured DRG [10]. The present study further exhibited that this upregulation was attributed to the peripheral nerve trauma-induced increase in C/EBPβ in the damaged DRG. Based on our findings, C/EBPβ was involved in transcriptionally activating the *Tlr7* gene in the damaged DRG neurons under neuropathic pain conditions.

C/EBPβ belongs to the C/EBPs family with six members that have been reported, which are basic-leucine zipper transcription factors that interact with CCAAT motifs present in numerous gene promoters. Similar to TFs like myeloid zinc finger [29,30], octamer transcription factor 1 [17,31], and runt-related transcription factor 1 [14], C/EBPβ expression can be regulated following peripheral nerve trauma. A previous study revealed that chronic sciatic nerve construction injury upregulated C/EBPβexpression at protein and mRNA levels in ipsilateral L3/4 DRGs [24]. However, whether C/EBPβ expression in intact/adjoining DRG was altered following peripheral nerve trauma is unclear. The present study demonstrated that SNL persistently enhanced the C/EBPβ protein level in the ipsilateral L4 DRG, rather than the intact/adjacent L3 DRG, ipsilateral and contralateral L4 DRGs. Based on these results, such increase only occurs in the damaged DRG neurons, as C/EBPβmRNA was identified in small, medium, and large DRG neurons, rather than satellite glial cells, of naive mice [24]. In addition, in the CCI mouse model, in the ipsilateral L3/4 DRGs, many neurons labeled with C/EBPβmRNA were positive for ATF3, the injury marker [24]. How peripheral nerve trauma results in the increased DRGC/EBPβ mRNA expression remains unclear, and this up-regulation may be associated with other TFs or enhanced RNA stability or epigenetic modifications, which should be further explored in the future.

C/EBPβ participates in peripheral nerve trauma-induced transcriptional activation of *Tlr7* promoter in injured DRG. TLRs play critical roles in stimulating innate immune responses against molecular patterns related to pathogens in mammals. TLR7 as a member of the TLRs is an endosomal innate immune sensor to recognize single-stranded RNAs [9]. In DRG neurons, TLR7 was positive for MrgprA3, CGRP, TRPA1, and TRPV1 [10–12], indicating that TLR7 may serve as an innovative pain mediator in nociceptive neurons. Indeed, we previously demonstrated that TLR7protein and mRNA expression increased in the damaged DRG neurons in the SNL mouse model [10]. Suppressing such increased expression mitigated the SNL-mediated nociceptive hypersensitivity in male and female mice's growth and maintenance [10]. Mimicking this upregulation strengthened the responses to cold/thermal/mechanical stimuli [10]. Mechanistically, the upregulated TLR7 triggered the nuclear factor kappa B (NF-κB) pathway by increasing nuclear import and phosphorylation of p65 in the damaged DRG neurons [10]. Thus, DRG upregulated TLR7 promotes neuropathic pain, possibly by the activation of NF-κB in the nociceptive primary sensory neurons. It is essential to understand how the *Tlr7* gene is transcriptionally activated in injured DRG post-SNL. The present study demonstrated that blocking DRG increased C/EBPβ by DRG microinjection of C/EBPβsiRNAattenuated SNL-mediated TLR7 up-regulation in damaged DRG. DRG overexpression of C/EBPβ elevated the expression of TLR7 in the injected DRG.

These *in-vivo* findings were further confirmed *in-vitro* in DRG neuronal culture. To our surprise, C/EBPβ siRNA microinjection had no significant difference to the baseline TLR7 or C/EBPβ protein expression of the sham group, even though siRNA showed high efficiency in cultured DRG neurons. The underlying cause of the above phenomenon is uncertain but might be due to its lower level that could not be further reduced by C/EBPβ siRNA at the dosage used in DRG normally. Additionally, C/EBPβ mRNA might have higher translational efficacy after knockdown, maintaining the normal baseline C/EBPβ protein expression in damaged DRG in the sham group. The present study also demonstrated C/EBPβ binding to the *Tlr7* promoter and increased this binding after SNL in injured DRG. Based on our results, C/EBPβ stimulated *Tlr7* promoter activation and co-expressed with TLR7mRNA in the individual DRG neurons. Overall, the above results illustrate that C/EBPβ has a vital function in the peripheral nerve trauma-induced increase of TLR7 in injured DRG.

C/EBPβ promotes neuropathic pain's genesis, likely through the triggered activation of *Tlr7* in injured DRG. The evidence from the present work and previous study [24] revealed that blocking DRG increased C/EBPβ and mitigated the development of nociceptive hypersensitivity caused by CCI or SNL. C/EBPβ Overexpression in DRG produced spontaneous pain and increased responses to mechanical, thermal, and cold stimulation [24]. The evidence indicates the participation of DRG increased C/EBPβ in nerve trauma-induced nociceptive hypersensitivity. As discussed, SNL-induced upregulation of DRG TLR7 mediates this participation. However, the involvement of other downstream targets of C/EBPβ cannot be excluded. For example, the previous study showed C/EBPβ role in peripheral nerve trauma-induced upregulation of euchromatic histone-lysine N-methyltransferase 2 in injured DRG [24]. Multiple mechanisms may mediate the role of increased C/EBPβ in DRG in neuropathic pain genesis.

Declarations

Authors' Contributions

Y.A. conceived the project, supervised all experiments, and edited the manuscript. L.H., J.C., B.C.J., J.J.Y., Y.X.T., and Y.A. assisted with experimental design. L.H. and J.C. carried out behavioral tests, surgery, and tissue collection. L.H. and B.C.J. performed molecular and biochemical experiments. L.H., J.C., B.C.J., and J.J.Y. analyzed the data. L.H. wrote the draft of the manuscript. Y.X.T. and Y.A. edited the manuscript. All authors read and discussed the manuscript.

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Data Availability

The data that support the results of this study are available from the corresponding author upon reasonable request.

Welfare of Animals

All procedures performed in studies involving animals were approved by the ethical standards of Institutional Animal Care and Use Committees of Zhengzhou University, Zhengzhou 450052, China. Mice received humane care following the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

Consent to Participate

Not applicable.

Consent for Publication

Yes

Conflict of Interest

The authors declare that they have no conflict of interest.

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Tables

Table 1 Primers used-

Names	Sequences
Single-cell RT-PCR	
Tlr7 F	5'-GGTATGCCGCCAAATCTAAA-3'
Tlr7 R	5'-GCTGAGGTCCAAAATTTCCA-3'
Cebp-β F	5'-CAAGCTGAGCGACGAGTACA-3'
Cebp-β R	5'-CAGCTGCTCCACCTTCTTCT-3'
Gapdh F	5'-TCGGTGTGAACGGATTTGGC-3'
Gapdh R	5'-TCCCATTCTCGGCCTTGACT-3'
ChIP RT-PCR	
Tlr7-promoter F	5'-AGGACAGGTTGCTTTATCAGGT-3'
Tlr7-promoter R	5'-TAACTTACACCACACGGGGG-3'
Luciferase	
Tlr7 F	5'-CGGGGTACCAACCTAAACCACACAGCCCC-3'
Tlr7 R	5'-CCCAAGCTTACAGAAAACCGAGACTCGCA-3'

RT: Reverse-transcription. F: Forward. R: Reverse

Table 2 Locomotor tests

Treatment groups	Locomotor function tests		
	Placing	Grasping	Righting
NC siRNA + Sham	5 (0)	5 (0)	5 (0)
Cebp-β siRNA + Sham	5 (0)	5 (0)	5 (0)
NC siRNA + SNL	5 (0)	5 (0)	5 (0)
Cebp-β siRNA + SNL	5 (0)	5 (0)	5 (0)
AAV5-GFP	5 (0)	5 (0)	5 (0)
AAV5-C/EBP-β	5 (0)	5 (0)	5 (0)

Values are means (SEM). n = 10 – 12 mice/group; 5 trials. NC: Negative control

Figures

Fig. 1

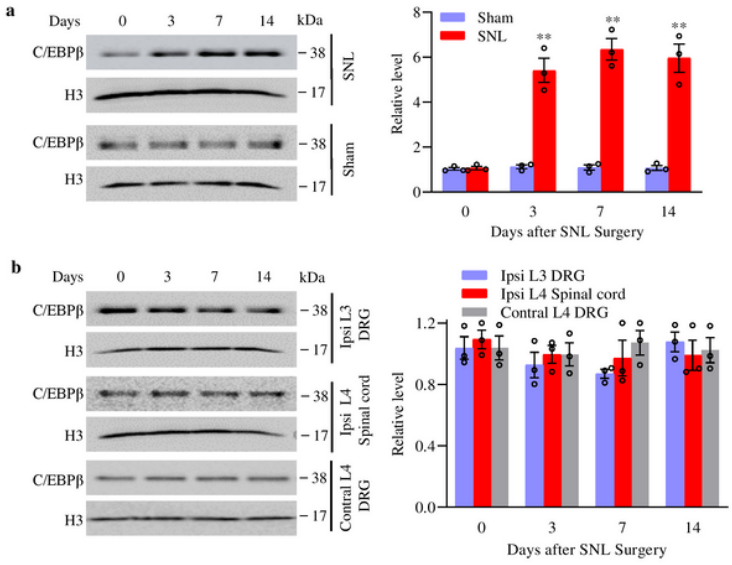

Figure 1

Peripheral nerve trauma-induced C/EBPβ protein increase in ipsilateral L4 DRG. (a) The protein level of C/EBPβ in ipsilateral L4 DRG in sham surgery or SNL mice. Typical Western blots (left panels) as well as densitometric analysis (right graphs). Three biological replicates were set (n = 12 mice) at every time

point for every group. **P < 0.01 vs. control (day 0) upon two-way ANOVA and post hoc Tukey test. (b) The protein level of C/EBPβ in ipsilateral L4 spinal cord, ipsilateral (intact) L3 DRG, and contralateral L4 DRG in SNL mice. Typical Western blots (left panels) as well as densitometric analysis (right graphs). Three biological replicates were set (n = 12 mice) at every time point. Two-way ANOVA and post hoc Tukey test.

Fig. 2

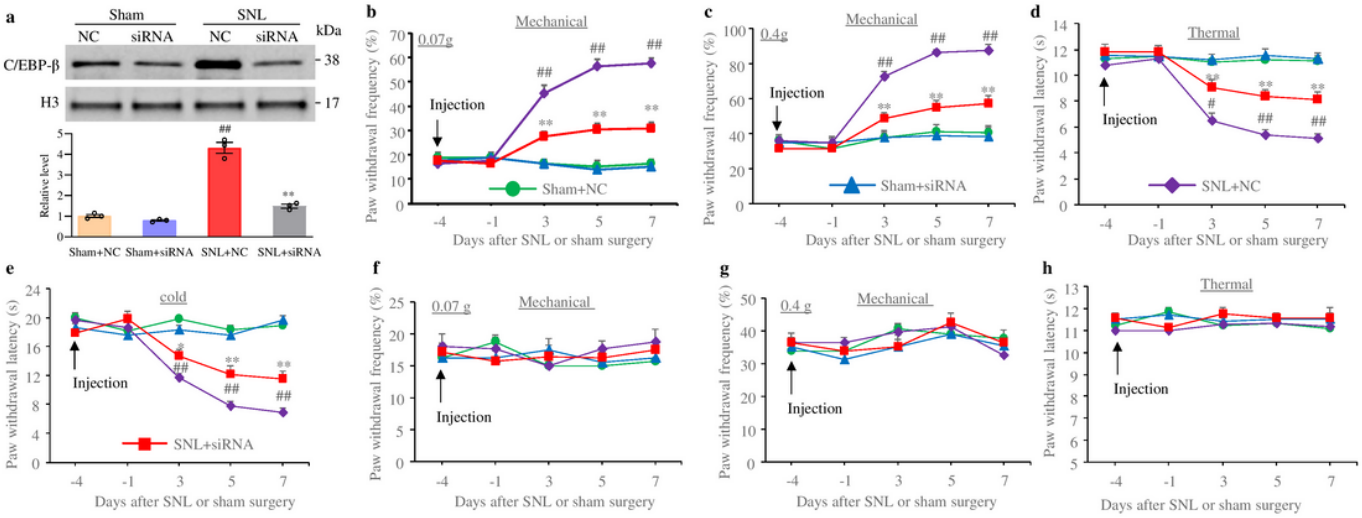

Figure 2

Blocking DRG C/EBPβ increase attenuates the occurrence of mouse neuropathic pain. siRNA: siRNA-C/EBPβ. NC: NC-siRNA. (a) The protein expression of C/EBPβ in ipsilateral L4 DRG at 7th day following sham surgery or SNL of treatment groups. Typical Western blots as well as densitometric analysis. Three biological replicates were set (n = 12 mice) for every group. One-way ANOVA and post hoc Tukey test. ##P < 0.01 versus the siRNA NC-microinjected sham mice. **P < 0.01 versus the siRNA NC-microinjected SNL mice. (b–h) Role of NC-siRNA or siRNA-C/EBPβ microinjection in ipsilateral L4 DRG in ipsilateral paw withdrawal responses upon mechanical (b, c, d, f), thermal (d, h) along with cold (e) stimuli in mice pre- and post-sham surgery or SNL. n = 12 mice in each group. Two-way ANOVA and repeated measurements as well as post hoc Tukey test. #P < 0.05 and ##P < 0.01 vs. the siRNA-negative control microinjected sham mice at the corresponding time points. **P < 0.01 versus siRNA NC-microinjected SNL mice at the corresponding time point.

Fig. 3

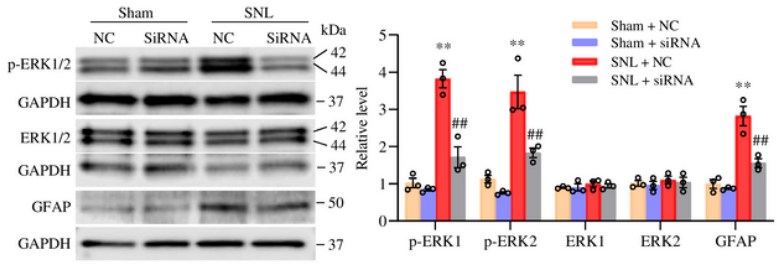

Figure 3

Role of siRNA-C/EBPβ pre-microinjection to ipsilateral L4 DRG in the central sensitization of dorsal horn, demonstrated by the SNL-mediated up-regulation of ERK1/2 phosphorylation (p-ERK1/2) together with GFAP expression in ipsilateral L4 dorsal horn at 7 days following sham surgery or SNL. Typical Western blots (left panels) together with densitometric analysis (right graphs) are displayed. n = 3 biological repeats (12 mice) per group. One-way analysis variance (ANOVA) and post hoc Tukey test. **P < 0.01 vs. relevant sham mice receiving NC-siRNA microinjection. ##P < 0.01 versus the corresponding siRNA NC microinjected SNL mice.

Fig. 4

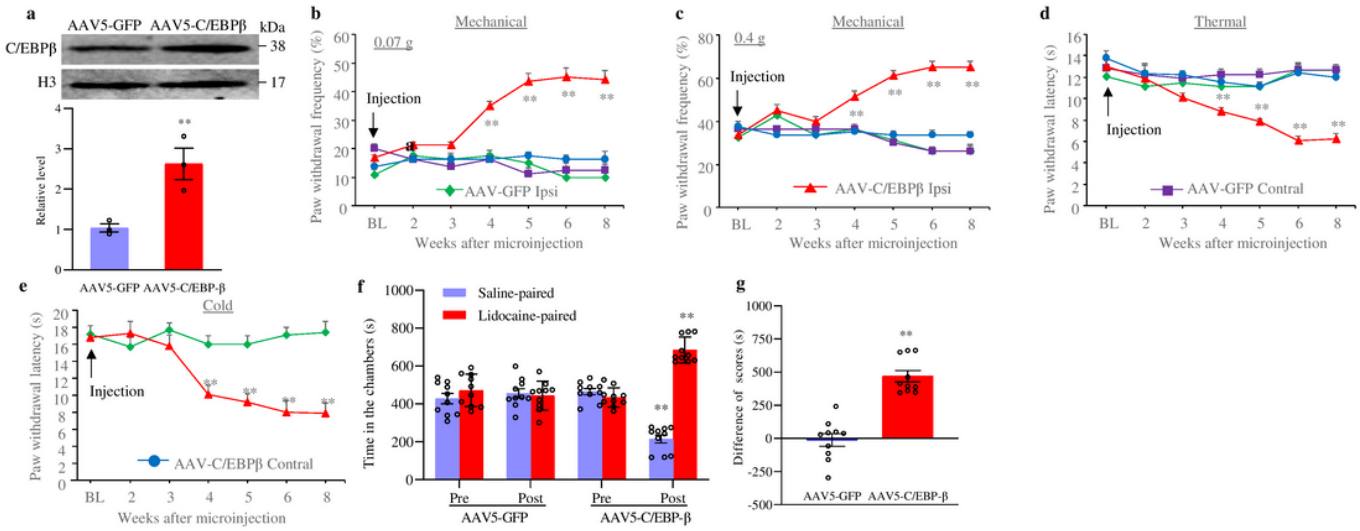

Figure 4

Overexpression of DRG C/EBPβ leads to symptoms of neuropathic pain. (a) The protein expression of C/EBPβ in ipsilateral L3/4 DRGs in week eight post-control AAV5-GFP or AAV5-C/EBPβ microinjection. Three biological replicates were set (n = 6 mice) for each group. **P < 0.01 vs. AAV5-GFP group upon unpaired two-tailed Student's t-test. (b–e) Ipsilateral and contralateral paw withdrawal responses upon mechanical (b, c), thermal (d), as well as cold (e) stimuli following microinjection in ipsilateral L3/4 DRG. n = 10 mice for each group. Two-way ANOVA and post hoc Tukey test versus the corresponding GFP group. BL: baseline. **P<0.01 versus ipsilateral control AAV5-GFP group at a specific time. (f, g) Role of control AAV5-GFP or AAV5-C/EBPβ microinjection in unilateral L3/4 DRGs in the persistent spontaneous pain evaluated using CPP paradigm. Post: post-conditioning. Pre: pre-conditioning. n = 10 mice for each group. Two-way ANOVA and post hoc Tukey test (f) or the independent, two-tailed Student's t-test (g). **P<0.01 versus the corresponding pre-conditioning.

Fig. 5

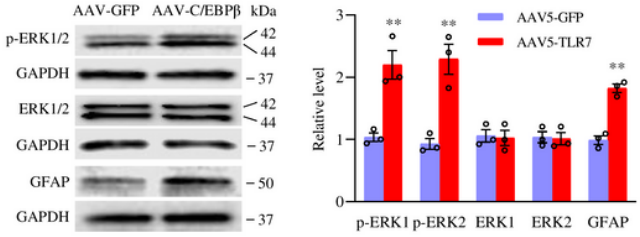

Figure 5

Role of overexpression of DRG C/EBPβ achieved by control AAV5-GFP or AAV5-C/EBPβ microinjection in unilateral L3/4 DRGs in ipsilateral L3/4 dorsal horn central sensitization, indicated by the increased ERK1/2 phosphorylation (p-ERK1/2) as well as GFAP expression in week eight following microinjection. Typical Western blots (left panels) as well as densitometric analysis (right graphs). Independent, two-tailed Student's t-test. Three biological replicates were set (n = 6 mice) for each group. **P < 0.01 vs related AAV5-GFP group.

Fig. 6

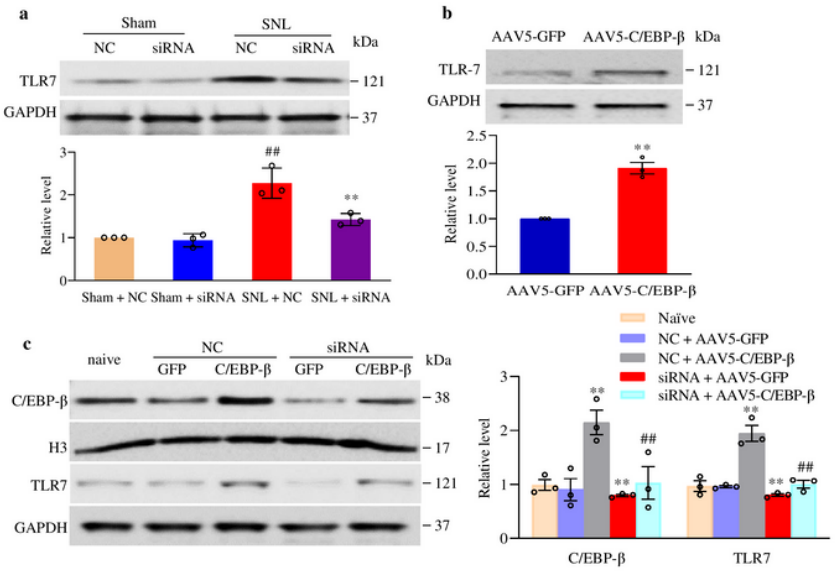

Figure 6

Participation of DRG increased C/EBPβ in nerve trauma-mediated TLR7 up-regulation in the damaged DRG. (a) TLR7 expression in ipsilateral L4 DRG in mice microinjected with NC-siRNA or C/EBPβ siRNA on day seven following sham surgery or SNL. Typical Western blots (left panels) as well as densitometric analysis (right graphs). Three biological replicates were set (n=12 mice for each group). ##P < 0.01 vs. sham surgery group given NC-siRNA microinjection. **P < 0.01 vs. SNL group given NC-siRNA microinjection upon one-way ANOVA and post hoc Tukey test. (b) TLR7 expression in L3/4 DRGs in week eight following control AAV5-GFP or AAV5-C/EBPβ microinjection. n=3 biological replicates (n = 6 mice for each group). **P<0.01 vs. AAV5-GFP group upon independent, two-tailed Student's t-test. (c) TLR7 protein expression in cultured mouse DRG neurons. Three biological replicates were set for each treatment. One-way analysis variance (ANOVA) and post hoc Tukey test. **P<0.01 vs related naïve group. ##P<0.01 vs. related AAV5-C/EBPβ plus C/EBPβ siRNA-treated group.

Fig. 7

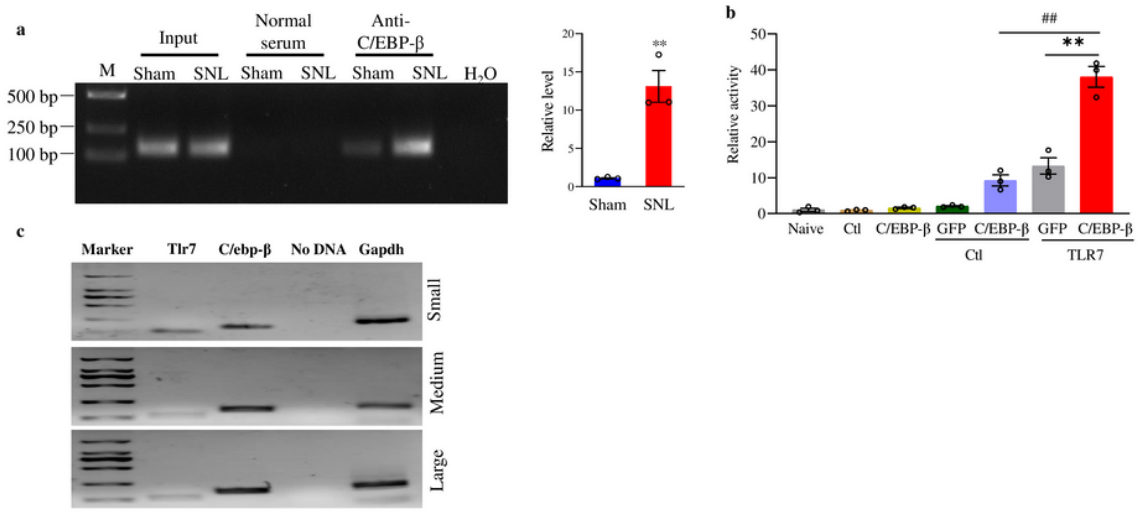

Figure 7

C/EBPβ binds to and triggers the Tlr7 promoter in ipsilateral L4 DRG after injury to the peripheral nerve. (a) On the 7th day after sham surgery or SNL, a fragment in the Tlr7 promoter was immunoprecipitated with the rabbit anti-C/EBPβ antibody in ipsilateral L4 DRG. M: ladder marker. Input: the whole Tlr7 mRNA fragment purified. Three biological replicates (n = 30 mice) in each group. **P<0.01 upon unpaired two-tailed Student's t-test compared with the sham group. (b) The activity of Tlr7 promoter in vectors-and siRNAs-transfected HEK-293T cells. Ctl: control vector (pGL3-Basic). Naive: untreated. GFP: Gfp-expressing pro-viral vector. C/EBP-β: full-length C/EBPβ-expressing pro-viral vector. TLR7: Tlr7 promoter-containing pGL3 reporter vector. n=3 replicates for each treatment. **P<0.01 compared with pGL3 Tlr7 vector (Tlr7) alone upon one-way ANOVA and post hoc Tukey test. (c) C/EBPβ mRNA was co-expressed with Tlr7 mRNA in lumbar DRG neurons of all sizes (small, moderate, large). Marker: ladder marker. n = 3 biological replicates.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CEBPbetaCPP.xlsx
- CEBPbetaKD.xlsx
- CEBPbetaOE.xlsx

- CEBPbetaProject.pzfx