The transcription factor CCAAT/enhancer-binding protein β in spinal microglia contributes to pre-operative stress-induced prolongation of postsurgical pain

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Abstract

Prolongation of postsurgical pain caused by pre-operative stress is a clinically significant problem, although the mechanisms are not fully understood. Stress can promote the pro-inflammatory activation of microglia, and the transcription factor CCAAT/enhancer-binding protein (C/EBP) β regulates pro-inflammatory gene expression in microglia. Therefore, we speculated that C/EBPβ in spinal microglia may have critical roles in the development of chronic postsurgical pain. Accordingly, in this study, we used a single prolonged stress (SPS) procedure and plantar incisions to evaluate the roles of C/EBPβ in postsurgical pain. Our experiments showed that SPS exposure prolonged mechanical allodynia, increased the expression of C/EBPβ and pro-inflammatory cytokines, and potentiated the activation of spinal microglia. Subsequently, microinjection of C/EBPβ siRNA attenuated the duration of SPS-prolonged postoperative mechanical allodynia and inhibited microglial activation in the spinal cord. Conversely, mimicking this increase in C/EBPβ promoted microglial activation via pretreatment with a pre-injection of AAV5-C/EBPβ, leading to prolongation of postsurgical pain. Overall, these results suggested that spinal microglia may play key roles in prolongation of postsurgical pain induced by pre-operative stress and that C/EBPβ may be a potential target for disease treatment.

Keywords

pre-operative stress, CCAAT/enhancer binding protein β, microglia, neuroinflammation, postsurgical pain

Introduction

Depression, anxiety, pre-operative pain catastrophizing, and other related pre-operative psychosocial factors have key roles in chronic postsurgical pain, which is defined as pain lasting for three or more months after surgery.[1,2] Exposure to psychological stressors can increase pain sensitivity, a conditioned called stress-induced hyperalgesia (SIH).[3] Furthermore, postoperative pain in incisional rats was shown to be delayed by exposure to a single prolonged stress (SPS) in our previous study.[4] Emerging evidence also suggests that persistent central nervous system (CNS) inflammation can be affected by stress, leading to SIH.[5]

Microglia are resident macrophages in the CNS and are responsible for propagation of neuro-inflammation, which is related to the development of chronic pain.[6] Overactivated microglia affect normal neurons by secreting pro-inflammatory factors (e.g. interleukin [IL]-1β, IL-6, and

tumor necrosis factor [TNF]-α), chemokines (e.g. C-C chemokine motif ligand 2 [CCL2], C-X-C chemokine motif ligand [CXCL] 9, and CXCL10), and oxidative metabolites, which are the main source of inflammatory mediators in the

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CNS.[7,8] We speculated that pre-operative stress may enhance the pro-inflammatory response of spinal microglia to subsequent peripheral injury, resulting in chronic postoperative pain.

CCAAT/enhancer binding protein β (C/EBPβ) participates in memory formation and synaptic plasticity in neurons and can regulate the activation of glia through pro-inflammatory gene expression.[9,10] Moreover, C/EBPβ also regulates cytokines, plasma membrane receptor proteins, and inflammation-related molecules in the inflammatory response.[11] As the key gene regulating microglial activation, the transcription factor C/EBPβ binds with the promoter and enhancer regions of cytokines and inflammatory genes.[12] For example, C/EBPβ negatively regulates early growth response 2 (EGR2); EGR2 overexpression promotes macrophage M2 polarization and inhibits the inflammatory response in a model of peritonitis, and lipopolysaccharide (LPS) treatment results in long-term downregulation of intracellular EGR2 and induces inflammatory activation.[13] Importantly, there are three potential binding sites for C/EBPβ in the EGR2 promoter region, and C/EBPβ has been shown to promote macrophage M1 polarization through binding to the EGR2 promoter region to inhibit EGR2 transcription and expression.[13] Knockdown of C/EBPβ using small interfering RNA (siRNA) resulted in EGR2 accumulation, thereby alleviating cellular inflammatory responses.[14] However, the mechanisms through which C/EBPβ regulates EGR2 to modulate post-surgical pain have not yet been reported.

Accordingly, in this study, we evaluated whether EGR2 regulated C/EBPβ-mediated neuro-inflammation in spinal microglia to contribute to chronic postsurgical pain after the SPS procedure. In addition, we assessed the value of C/EBPβ as a therapeutic target for chronic postsurgical pain induced by pre-operative stress by suppression of spinal microglial activation.

Materials and methods

Experimental animals

Adult male Sprague-Dawley rats (weighing 250–300 g) were bred locally at the Laboratory Animal Centre of Drum Tower Hospital. The rats were housed under conditions of 50% humidity and a 12-h alternating light:dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing University.[15]

SPS procedure and incisional surgery

The SPS procedure was carried out as described by Liberzon et al.[16] Animals were confined in holders for 2 h and were then forced to swim for 20 min individually in a clear acrylic cylinder filled two-thirds full with water (24°C). The rats were allowed to rest for 15 min after swimming and were then anesthetized by inhalation of an ether anesthetic. Control rats were left untreated and were observed in another room.

The incisional surgery was performed as described by Brennan et al. under sterile conditions.[17] Rats were anesthetized by inhalation of sevoflurane using a nose mask. A 1-cm longitudinal incision was made through the skin and fascia on the right hind paw. The plantaris muscle was elevated using forceps, keeping the muscle origin and insertion intact. The wound was sutured with two 5–0 nylon mattress sutures and treated with aureomycin ointment.

Nociceptive behavioral tests

The paw withdrawal threshold to mechanical stimuli was tested with a set of von Frey filaments. Before each test, rats were placed in plastic boxes with a wire mesh bottom, and paw withdrawal mechanical threshold (PWMT) was assessed using the "up-and-down" method. The right hind paw was positioned by the von Frey filament adjacent to the wound for 6–8 s. Paw lifting or licking of the paw following stimulation was defined as a positive response. The data were recorded and analyzed using the method described by Chaplan et al.[18]

Intrathecal microinjection

According to a previously described method, intrathecal injections were administered through implanted lumbar intrathecal catheters.[19] Briefly, under anesthesia with sevoflurane and after sterilization of the skin, rats were immobilized and a 2-cm longitudinal incision was made between vertebrae L5 and L6. The spinal dura mater was pierced using a syringe needle, and a polyethylene catheter (PE-10) was implanted into the lumbar enlargement. Injection of lidocaine (2%, 20 μL) through the catheter was used to confirm correct intrathecal placement. Based on the study protocol, 20 μL drugs or vehicle was injected, and the catheter was then flushed with 20 μL saline. All rats were observed after intrathecal administration, and rats exhibiting poor grooming or postoperative neurological deficits were excluded from further analyses.

We purchased C/EBPβ siRNA (cat. No. sc-29862; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and its negative control siRNA (cat. No. sc-44230) for siRNA injection. As a delivery vehicle, TurboFect in vivo transfection reagent (Thermo Fisher Scientific, MA, USA) was performed to improve delivery and prevent degeneration of siRNA.[20,21] Full-length C/EBPβ cDNA was gel-purified and ligated into proviral plasmids after digestion with XbaI/BamHI. Liposomes 5 μl and siRNA 5 μl were separately diluted with Opti-MEM 250 μl and then they were mixed evenly at room temperature for 20 min. After removing the culture medium, Opti-MEM 500 μL and lipo-siRNA mixture were added into the culture plate. After 48 h of transfection, the fluorescence content was observed to determine the transfection efficiency of siRNA. The cell RNA and protein were extracted and the transfection efficiency of them was detected by qPCR and western blotting after 72 h. Under control of the cytomegalovirus promoter, the resulting plasmid expressed C/EBPβ; a

plasmid expressing enhanced fluorescent protein (EGFP) was used as a control. Recombinant adeno-associated virus 5 (AAV5) viral particles harboring these two cDNAs were produced at the UNC Vector Core.

Western blotting

After being deeply anesthetized with sevoflurane, the L3–L5 spinal cords of rats were separated and homogenized in lysis buffer. The homogenate was centrifuged (4°C) at 13,000 r/min for 10 min. The protein concentration was determined by the BCA Protein Assay Kit, following the manufacturer's instructions. Proteins and a molecular weight marker were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore Corporation, MA, USA). The filter membranes were blocked with 5% skimmed milk for 1 h at room temperature and incubated overnight at 4°C with the following primary antibodies: C/EBPβ (1:500; Santa Cruz Biotechnology), EGR2 (1:1000; Abcam, UK), IL-1β (1:100; Abcam), CCL2 (1:1000; Abcam), TNF-α (1:500; Santa Cruz Biotechnology), and β-actin overnight. The membranes were then rinsed with TBST and incubated with the secondary antibody at room temperature for 2 h in the dark. Protein bands were visualized using an enhanced chemiluminescence solution (Millipore). The gray value of each band was measured and quantified using NIH ImageJ software.

Immunohistochemistry

Under sevoflurane deep anesthesia, the rats were perfused through the heart with paraformaldehyde, and the L3–L5 segments of the lumbar spinal cord were quickly removed and embedded in paraffin. The paraffin-embedded tissues were cut transversely and mounted on glass slides. Sections were washed with phosphate-buffered saline (PBS) and incubated overnight at 4°C with a primary antibody targeting the microglia-specific marker ionized calcium binding adaptor molecule 1 (IBA-1; 1:2000; Abcam). Hematoxylin and eosin staining was used to counterstain the sections. Microglial activation of the spinal cord was quantified using ImageJ software by counting IBA-1-positive cells.[22]

Immunofluorescence staining

Rats were terminally anesthetized with sevoflurane and perfused through the heart with saline followed by 500 mL ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. L3–L5 spinal cords of rats were removed and transferred to 30% sucrose for 48 h. The tissues were transversely cut into sections using a sliding microtome. After washing with PBS, sections were incubated with the following primary antibodies: anti-IBA-1 (microglial marker; 1:500; Wako, Japan), anti-C/EBPβ (1:500; Santa Cruz Biotechnology), and anti-CD86 (microglial M1 marker; 1:500; Abcam). Sections were then incubated with appropriate secondary antibodies, and images were acquired at 200× magnification using a confocal microscope (Leica TCS SP2; Leica, Wetzlar, Germany).

Statistical analysis

All data were expressed as means ± standard deviations. Two-way repeated measures analysis of variance was used to analyze PWMT. Comparisons of nonparametric data obtained from western blots were analyzed using Kruskal–Wallis test. When significance was observed, the sources of differences were determined using Bonferroni post-hoc tests. All statistical analyses were carried out using SPSS 19.0, and differences among groups were considered statistically significant when the p value was less than 0.05.

Results

The SPS procedure induced postoperative hyperalgesia and prolonged mechanical allodynia and elevated the expression of microglia in the spinal cord

To assess SPS-related pain behaviors, PWMT was measured on days 1, 3, 5, 7, 10, 14, 17, 21, and 28 after incision surgery. The time schedule and experimental design are shown in Figure 1(a). There were no significant differences in baseline PWMT values between groups before the SPS procedure. Compared with baseline and control rats, rats with incisional surgery showed lower PWMT values for 3 days, and a decrease in PWMT was observed for 14 days in rats exposed SPS. Furthermore, rats showed lower PWMT values for up to 21 days after the SPS procedure and incisional surgery (Figure 1(b)). Thus, we concluded that pre-operative SPS induced prolongation of postsurgical pain.

Next, the expression of microglia in the spinal cord was investigated during the development of pre-operative SPS-prolonged incision-induced mechanical allodynia. Immunohistochemical analyses demonstrated that the expression of cells positive for IBA-1 (a protein marker of microglial activation) in rats subjected to the SPS procedure and incisional surgery was significantly increased on day 3 after surgery compared with that in rats subjected to incision only (Figures 1(c) and (d)). Furthermore, double fluorescence intensity of C/EBPβ and microglia in the spinal cord in rats subjected to the SPS procedure and incisional surgery was significantly potentiated compared with that in rats subjected to incisional surgery on day 3 after surgery (Figures 2(a) to (c)).

The M1-like phenotype of microglia is characterized by the production of pro-inflammatory mediators as well as increased expression of surface markers, such as CD86, which fuel the inflammatory process.[23] Therefore, we next examined the phenotype of microglia during SPS-prolonged mechanical allodynia. Immunofluorescence analyses showed that microglia in rats subjected to the SPS procedure and incisional surgery showed high expression of M1 markers
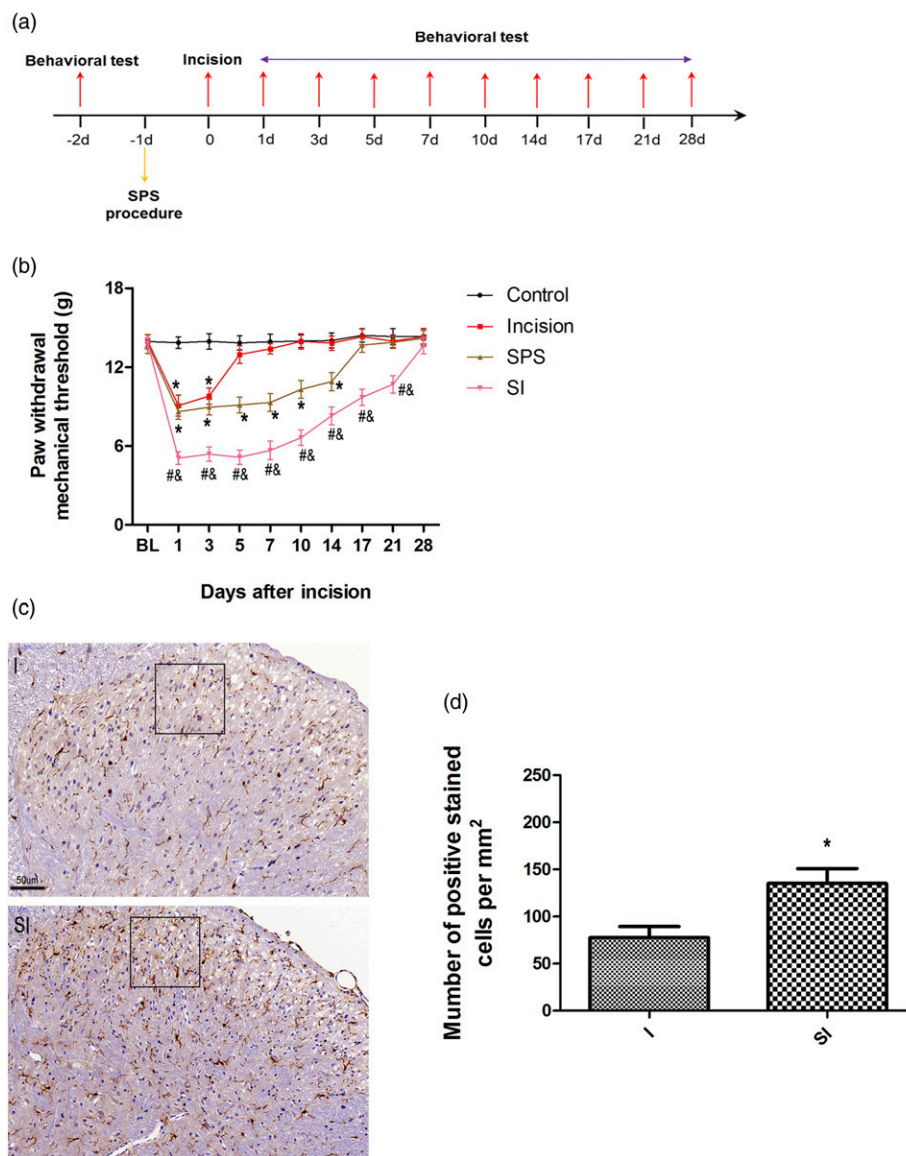
Figure 1. SPS procedure induced postoperative hyperalgesia and prolonged mechanical allodynia, as well as elevated expression of microglia in the spinal cord. (a) The experimental design and time schedule. Behavioral tests were conducted on the day before SPS (baseline) and on days 1, 3, 5, 7, 10, 14, 17, 21, 28 after incision surgery. (b) Preoperative SPS prolonged incision-induced mechanical allodynia (n=6 per group). (c) Immunohistochemical staining for the number of IBA-1-positive cells (a protein marker of microglial activation) in spinal cord dorsal horn. (d) The numbers of IBA-1-positive stained cells in Group SI significantly increased on day 3 after surgery compared with Group I (n=6 per group). Group Control: rats underwent sham SPS and sham incision surgery; Group Incision: rats underwent an incisional surgery with sham SPS procedure; Group SPS: rats underwent SPS procedure with sham incision surgery; Group SI: rats underwent an incisional surgery 1 d after the SPS procedure. *p < 0.05 compared with Group Control; #p < 0.05 compared with Group Incision; &p < 0.05 compared with Group SPS.

compared with that in rats subjected to incision only on day 3 after surgery (Figures 2(d) and (e)).

Pre-operative SPS influenced the expression of C/EBPβ and EGR2 and potentiated the pro-inflammatory response after incision surgery

The pro-inflammatory response of microglia to subsequent pro-inflammatory challenges is enhanced by exposure to stress.[24] As the key gene regulating microglial activation, we further examined C/EBPβ levels in the spinal cord following maintenance of SPS-prolonged postoperative mechanical allodynia. The expression levels of C/EBPβ and EGR2 proteins in the spinal cord were evaluated using western blotting on day 3 and on day 21 after surgery. The results demonstrated that rats subjected to the SPS procedure and incisional surgery showed higher expression of C/EBPβ compared with the other groups; by contrast, EGR2 protein levels in rats subjected to the SPS procedure and incisional surgery were significantly decreased after surgery (Figures 3(a) to (c)).
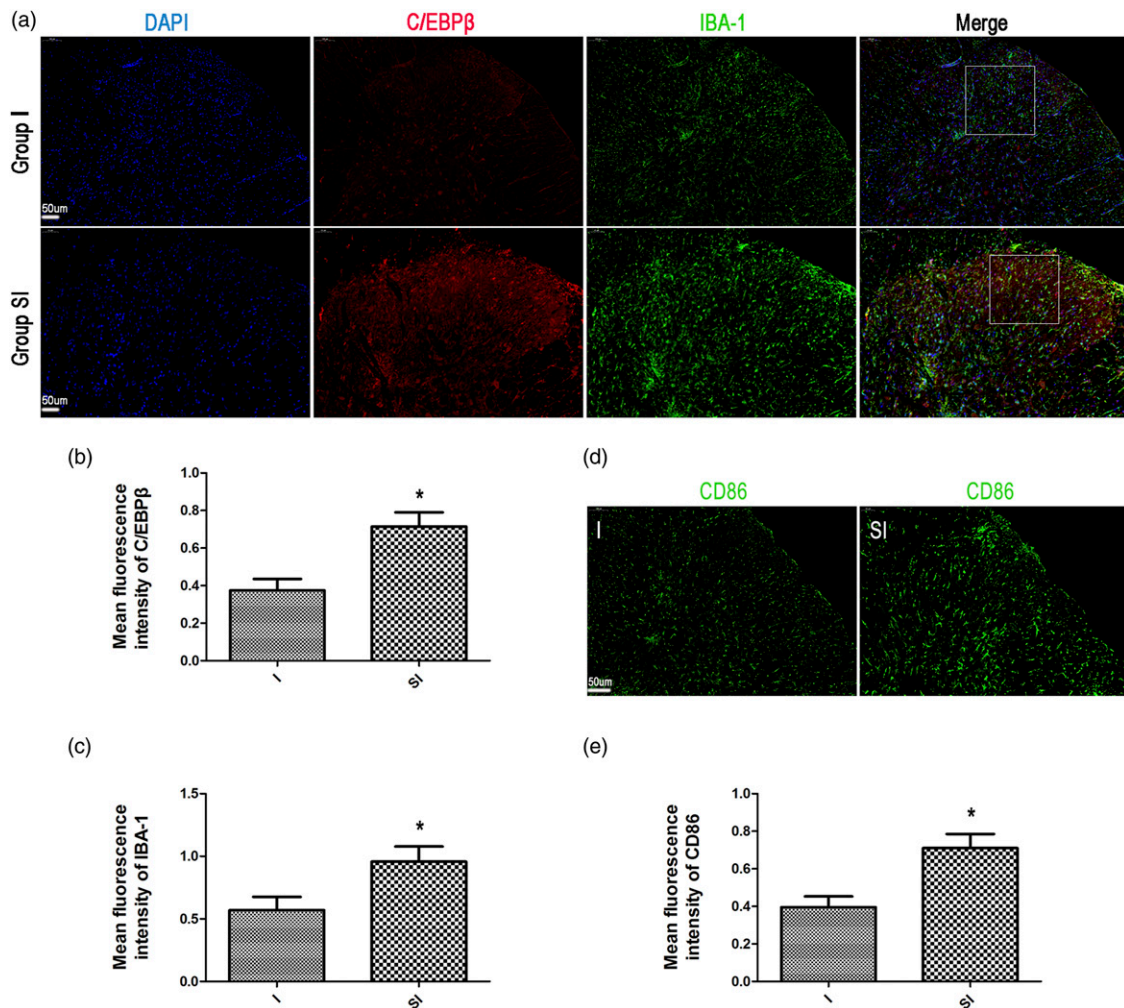
Figure 2. SPS procedure increased the expression of C/EBPβ and microglia after incision surgery in spinal cord. (a) Double-immunofluorescence labeling for C/EBPβ (red) and IBA-1-positive cells (green, a microglia marker) in the ipsilateral spinal cord. (b–c) The fluorescence intensity of C/EBPβ and IBA-1 in Group SI significantly increased compared with Group I on day 3 after surgery (n=6 per group). (d) Immunofluorescence labeling for the CD86-positive microglia (a protein marker of M1 microglial activation) in spinal cord dorsal horn. (e) Microglia in Group SI showed high expression of M1 markers compared with Group I on day 3 after surgery (n=6 per group). Group I: rats underwent an incisional surgery with sham SPS procedure; Group SI: rats underwent an incisional surgery 1 d after the SPS procedure. *$p < 0.05$ compared with Group I.

Next, the expression levels of the pro-inflammatory cytokines IL-1β, CCL2, and TNF-α were measured using western blotting. Rats subjected to the SPS procedure and incisional surgery showed upregulation of IL-1β, CCL2, and TNF-α compared with those in the other groups on day 3 and on day 21 after surgery (Figures 3(d) to (g)).

Microinjection of C/EBPβ siRNA attenuated SPS-prolonged postoperative mechanical allodynia and suppressed SPS-potentiated microglia activation in the spinal cord

According to the above results, spinal microglia were stimulated by the SPS procedure, which prolonged postoperative mechanical allodynia. In previous studies, C/EBPβ

has been shown to regulate cytokines, plasma membrane receptor proteins, and inflammation-related molecules during the inflammatory response.[25,26] In the current study, we found that C/EBPβ exhibited higher expression in rats subjected to the SPS procedure and incisional surgery. Therefore, to examine the active role of C/EBPβ in SPS-prolonged postoperative mechanical allodynia in the spinal cord, we microinjected siRNA targeting C/EBPβ into the spinal cord and examined whether this could block the SPS-induced increase in C/EBPβ expression. The time schedule and experimental design are shown in Figure 4(a). Microinjection of C/EBPβ siRNA promoted the recovery from pre-operative SPS-induced prolongation of postsurgical pain (Figure 4(b)). As expected, there were no significant differences in PWMT values between the vehicle-injected group and the negative siRNA-injected group. These findings suggested that the
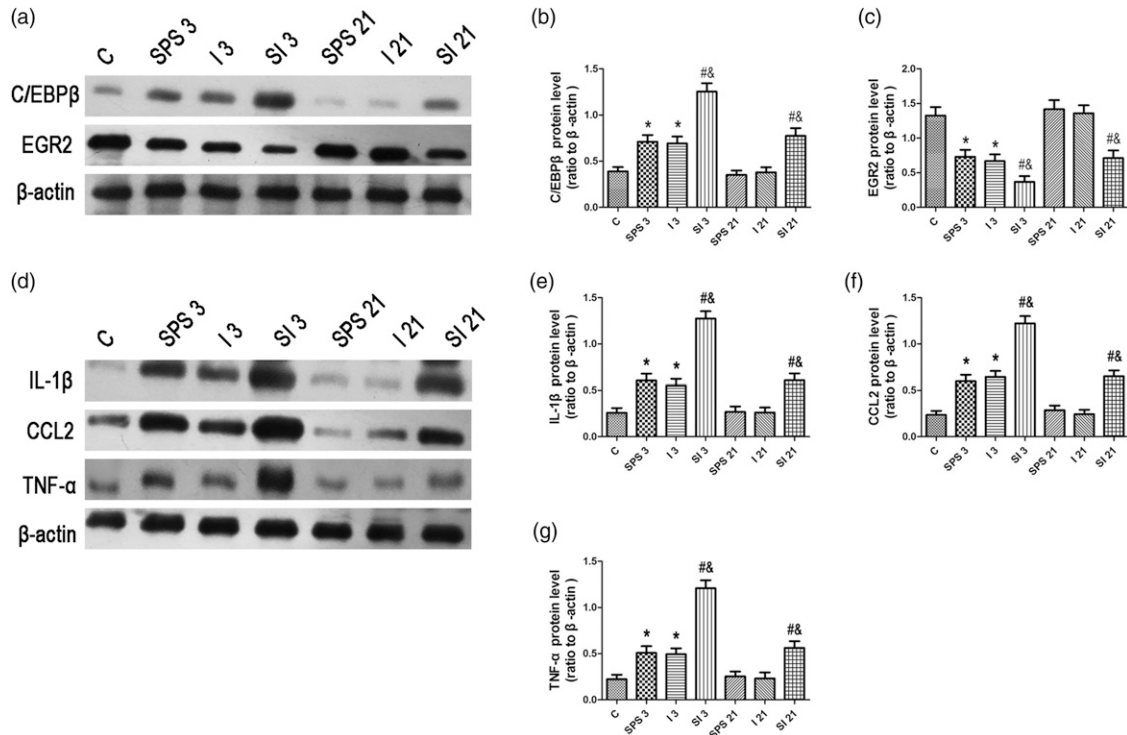
Figure 3. Preoperative SPS influenced the expression of C/EBPβ and EGR2 and elevated the expression of proinflammatory cytokines after incision surgery in the spinal cord. (a) Western blotting for the expression of C/EBPβ and EGR2 protein in spinal cord dorsal horn. (b–c) Group SI showed higher expression of C/EBPβ compared with the other groups, by contrast, EGR2 protein levels in Group SI were significantly decreased on days 3 and 21 after surgery (n=6 per group). (d) Western blotting for the proinflammatory cytokines expression of IL-1β, CCL2 and TNF-α protein in spinal cord dorsal horn. (e–g) Group SI showed upregulation of IL-1β, CCL2 and TNF-α protein compared with those in the other groups on day 3 and on day 21 after surgery (n=6 per group). Group C: rats underwent sham SPS and sham incision surgery; Group I: rats underwent an incisional surgery with sham SPS procedure; Group SPS: rats underwent SPS procedure with sham incision surgery; Group SI: rats underwent an incisional surgery 1 d after the SPS procedure. *$p < 0.05$ compared with Group C; #$p < 0.05$ compared with Group SPS; &$p < 0.05$ compared with Group I.

duration of postsurgical pain after SPS could be shorten by microinjection of C/EBPβ siRNA.

We further investigated whether blocking C/EBPβ affected the expression of microglia in the spinal cord. Immunohistochemical analyses demonstrated that the expression of IBA-1-positive cells in the dorsal horn was significantly decreased in rats subjected to postinjection of C/EBPβ siRNA (Figures 4(c) and (d)).

Microinjection of C/EBPβ siRNA suppressed SPS-potentiated C/EBPβ activation and pro-inflammatory cytokine expression after incision surgery

Next, spinal cords were collected after microinjection of C/EBPβ siRNA, and the expression levels of C/EBPβ and EGR2 proteins were evaluated using western blotting. Pre-injection of C/EBPβ siRNA significantly blocked SPS-induced increases in C/EBPβ protein and markedly increased the amounts of EGR2 protein (Figures 5(a) to (c)). Furthermore, western blotting revealed marked decreases in

IL-1β, CCL2, and TNF-α protein levels in the rats subjected to pre-injection of C/EBPβ siRNA compared with the rats subjected to the SPS procedure and incisional surgery (Figures 5(d) to (g)). These findings suggested that spinal C/EBPβ may have important roles in the initiation of SPS-induced spinal inflammatory responses and that blocking C/EBPβ activation enhanced the recovery from SPS-induced prolongation of postsurgical pain.

Mimicking the SPS-induced increase in C/EBPβ in the spinal cord prolonged postsurgical pain symptoms and potentiated microglial activation

Next, we aimed to clarify whether mimicking the SPS-induced increase in C/EBPβ in the spinal cord by intrathecal microinjection of AAV5-C/EBPβ altered nociceptive thresholds in rats subjected to incisional surgery. The time schedule and experimental design are shown in Figure 6(a). Notably, injection of AAV5-C/EBPβ yielded lower PWMT values and prolonged the duration of postsurgical pain
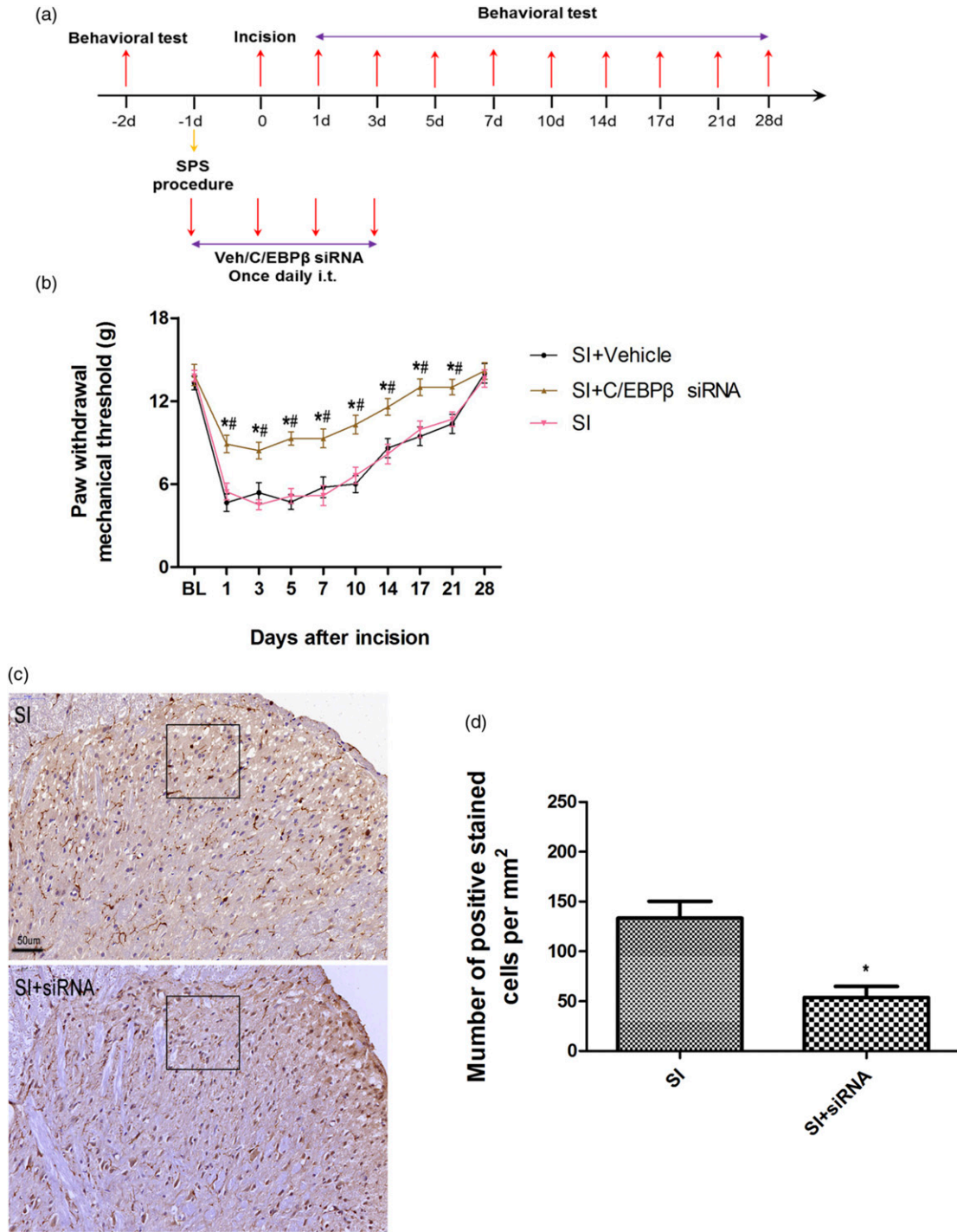
Figure 4. Perioperative inhibition of the C/EBPβ attenuated SPS-prolonged postoperative mechanical allodynia and suppressed SPS-potentiated microglia activation in the spinal cord. (a) The experimental design and time schedule. (b) Perioperative microinjection of siRNA targeting C/EBPβ promoted the recovery from pre-operative SPS-induced prolongation of postsurgical pain ($n=6$ per group). C/EBPβ siRNA was intrathecally injected once daily for consecutive 5 days (30 min before SPS exposure to 3 days after incision). (c) Immunohistochemical staining for the number of IBA-1-positive cells in spinal cord dorsal horn. (d) Immunohistochemical analyses demonstrated that the expression of IBA-1-positive cells in the dorsal horn was significantly decreased in Group SI+C/EBPβ siRNA ($n=6$ per group). Group SI: rats underwent an incisional surgery 1 d after the SPS procedure; Group SI+C/EBPβ siRNA: rats underwent an incisional surgery 1 d after the SPS procedure and underwent microinjection of C/EBPβ siRNA once daily for consecutive 5 days; Group SI+Vehicle: rats underwent an incisional surgery 1 d after the SPS procedure and underwent microinjection of vehicle once daily for consecutive 5 days *$p < 0.05$ compared with Group SI; #$p < 0.05$ compared with Group SI+Vehicle.

compared with those in rats subjected to incision only (Figure 6(b)).

We then confirmed whether mimicking the SPS-induced increase in C/EBPβ influenced microglial activation in the spinal cord. Rats injected with AAV5-C/EBPβ and subjected to incisional surgery displayed obvious increases in IBA-1-positive cells in the dorsal horn (Figures 6(c) and (d)). By contrast, rats subjected with incisional surgery and injected with the AAV5-EGFP control did not exhibit significant differences in microglia compared with rats subjected to incision only. These findings indicated that mimicking the SPS-induced increase in C/EBPβ prolonged postsurgical pain and activated spinal microglia.

Mimicking the SPS-induced increase in C/EBPβ in the spinal cord potentiated C/EBPβ activation and pro-inflammatory cytokine expression

To further assess the effects of C/EBPβ and EGR2 expression on SPS-induced postsurgical pain, AAV5-C/EBPβ was administered through intrathecal injection in rats subjected to incisional surgery. Western blotting revealed that pre-injection of AAV5-C/EBPβ significantly increased C/EBPβ protein levels but markedly reduced EGR2 protein levels (Figures 7(a) to (c)). When compared with rats subjected to incision only, rats injected with AAV5-C/EBPβ and subjected to incisional surgery showed marked increases in the pro-inflammatory cytokines IL-1β, CCL2, and TNF-α (Figures 7(d) to (g)). Thus, microinjection of AAV5-C/EBPβ into the spinal cord potentiated C/EBPβ activation and pro-inflammatory cytokine expression.

Discussion

In our study, we demonstrated that pre-operative SPS prolonged postoperative pain and promoted the pro-inflammatory response of microglia in the spinal cord. Moreover, C/EBPβ in spinal microglia and enhancement of pro-inflammatory cytokine expression played important roles in SPS-induced prolongation of postsurgical pain, and microinjection of C/EBPβ siRNA attenuated the duration of SPS-prolonged postoperative mechanical allodynia and suppressed spinal microglial activation. Finally, we showed that mimicking the SPS-induced increase in C/EBPβ in the spinal cord prolonged postsurgical pain symptoms and potentiated microglial activation. These findings supported the hypothesis that stress-induced C/EBPβ in the spinal cord triggered the excitability of microglia, promoting the prolongation of postsurgical pain.

Activation of microglia to a pro-inflammatory immunophenotype can be facilitated by exposure to some stressors.[27] Microglia activation and neuro-inflammatory responses to LPS were found to increase in rats exposed to chronic variable stress.[28] Furthermore, as shown in a study by Alexander et al., in a model of restraint stress-induced exacerbation of neuropathic pain, previous exposure to stress accelerated the activation of spinal microglia.[29] In response to changes in the environment, microglia maintain tissue homeostasis and exhibit diverse phenotypes.[30] Additionally, following injury, microglia become polarized towards a pro-inflammatory M1 phenotype and then produce pro-inflammatory cytokines, express high levels of inducible nitric oxide, and present antigen.[31,32] In the current study, we showed that pre-operative SPS induced postoperative hyperalgesia, prolonged mechanical allodynia, and elevated the expression of M1 microglia and the pro-inflammatory cytokines IL-1β, CCL2, and TNF-α in the spinal cord. Accordingly, the pro-inflammatory response of spinal microglia was enhanced by exposure to the SPS procedure, which aggravated and prolonged postoperative pain.

As a member of the C/EBP subfamily of bZIP transcription factors, C/EBPβ has roles in cell proliferation, differentiation, and the inflammatory response.[33,34] Putative C/EBPβ consensus sequences are present in the promoters of many pro-inflammatory genes, which upregulates the response to pro-inflammatory stimuli.[35-38] Previous studies have revealed that multiple genes related to cell growth and differentiation are regulated by EGR proteins[39-41] and that knockout of EGR2 leads to a lethal autoimmune syndrome related to excessive systemic pro-inflammatory cytokines.[42] Moreover, Tatyana showed that EGR2 is involved in the polarization and plasticity of macrophages and high levels of C/EBPβ negatively regulate EGR2 in M1 macrophages.[13] In the current study, SPS increased the expression of C/EBPβ protein and decreased the expression of EGR2 protein in rats subjected to incisional surgery. These findings indicated that stress may induce C/EBPβ-dependent modulation of microglia M1 polarization by blocking EGR2 transcription, thereby potentiating the pro-inflammatory response and promoting the duration of SPS-prolonged postoperative mechanical allodynia.

C/EBPβ may have regulatory roles in pro-inflammatory cytokine expression in neuro-inflammation, and our results showed that increased expression of C/EBPβ in the spinal cord following pre-operative stress-induced pain chronification after surgery. Thus, C/EBPβ may be a treatment target for SPS-prolonged postoperative allodynia. To further elucidate the role of C/EBPβ, we blocked the SPS-induced increase in C/EBPβ by microinjection of siRNA into the spinal cord. Our results revealed that microinjection of C/EBPβ siRNA alleviated pre-operative SPS-induced postsurgical pain, markedly reduced the abundance of C/EBPβ protein, and increased the expression of EGR2 protein, supporting that C/EBPβ siRNA induced these effects. Overall, these results were consistent with previous studies of C/EBPβ in neuro-inflammation. Indeed, the expression of C/EBPβ was shown to be increased in a model of chronic constriction injury, resulting in peripheral nerve trauma in the dorsal root ganglion, and the development and maintenance
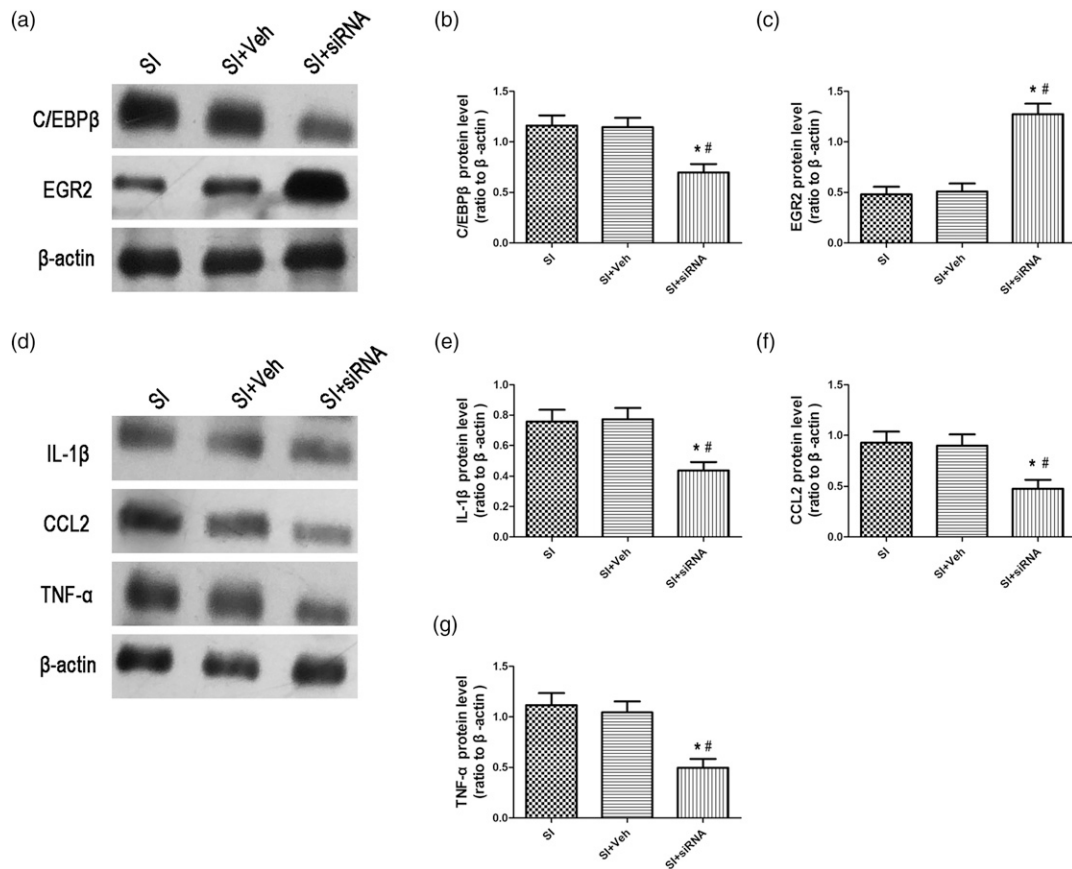
Figure 5. Microinjection of C/EBPβ siRNA suppressed SPS-potentiated C/EBPβ activation and pro-inflammatory cytokine expression after incision surgery. (a) Western blotting for the expression of C/EBPβ and EGR2 protein in spinal cord dorsal horn. (b–c) Pre-injection of C/EBPβ siRNA significantly blocked SPS-induced increases in C/EBPβ protein and markedly increased the amounts of EGR2 protein (n=6 per group). (d) Western blotting for the proinflammatory cytokines expression of IL-1β, CCL2 and TNF-α protein in spinal cord dorsal horn. (e–g) Results revealed IL-1β, CCL2, and TNF-α protein levels in Group SI+C/EBPβ siRNA marked decreased compared with those in other groups (n=6 per group). Group SI: rats underwent an incisional surgery 1 d after the SPS procedure; Group SI+siRNA: rats underwent an incisional surgery 1 d after the SPS procedure and underwent microinjection of C/EBPβ siRNA once daily for consecutive 5 days; Group SI+Veh: rats underwent an incisional surgery 1 d after the SPS procedure and underwent microinjection of vehicle once daily for consecutive 5 days *p < 0.05 compared with Group SI; #p < 0.05 compared with Group SI+Veh.

of mechanical pain hypersensitivities could be mitigated by blocking this increase in C/EBPβ.[43] In addition, we then determined whether intrathecal microinjection of AAV5-C/EBPβ altered nociceptive thresholds in rats subjected to incisional surgery. We found that injection of AAV5-C/EBPβ resulted in lower PWMT values and prolonged the duration of postsurgical pain, accompanied by increased expression of C/EBPβ and decreased expression of EGR2 protein. These findings suggested that C/EBPβ may directly negatively regulate EGR2 expression in the spinal cord after SPS-prolonged postoperative allodynia.

A series of studies suggested that microglial activation may be involved in the pathogenesis of chronic pain.[44-46] According to these results, stimulation of spinal microglia using the SPS procedure prolongs postoperative mechanical allodynia. Moreover, in the current study, pre-operative SPS caused acute postoperative pain to progress to chronic pain

from 3 to 21 days after surgery. Thus, suppression of microglial activation by inhibition of C/EBPβ may promote recovery from pre-operative SPS-induced prolongation of postsurgical pain. Consistent with this assumption, microinjection of C/EBPβ siRNA markedly suppressed the expression of microglia detected in the dorsal horn. Additionally, rats injected with C/EBPβ siRNA showed lower levels of the pro-inflammatory cytokines IL-1β, CCL2, and TNF-α than rats subjected to the SPS procedure and incisional surgery. Furthermore, we found that rats injected with AAV5-C/EBPβ and subjected to incisional surgery showed significant increases in the expression of microglia in the dorsal horn and exhibited potentiation of pro-inflammatory cytokine. Based on these findings, we concluded that exposure to SPS regulated C/EBPβ in spinal microglia to modulate pro-inflammatory responses, thereby contributing to exacerbation and prolongation of postoperative pain.
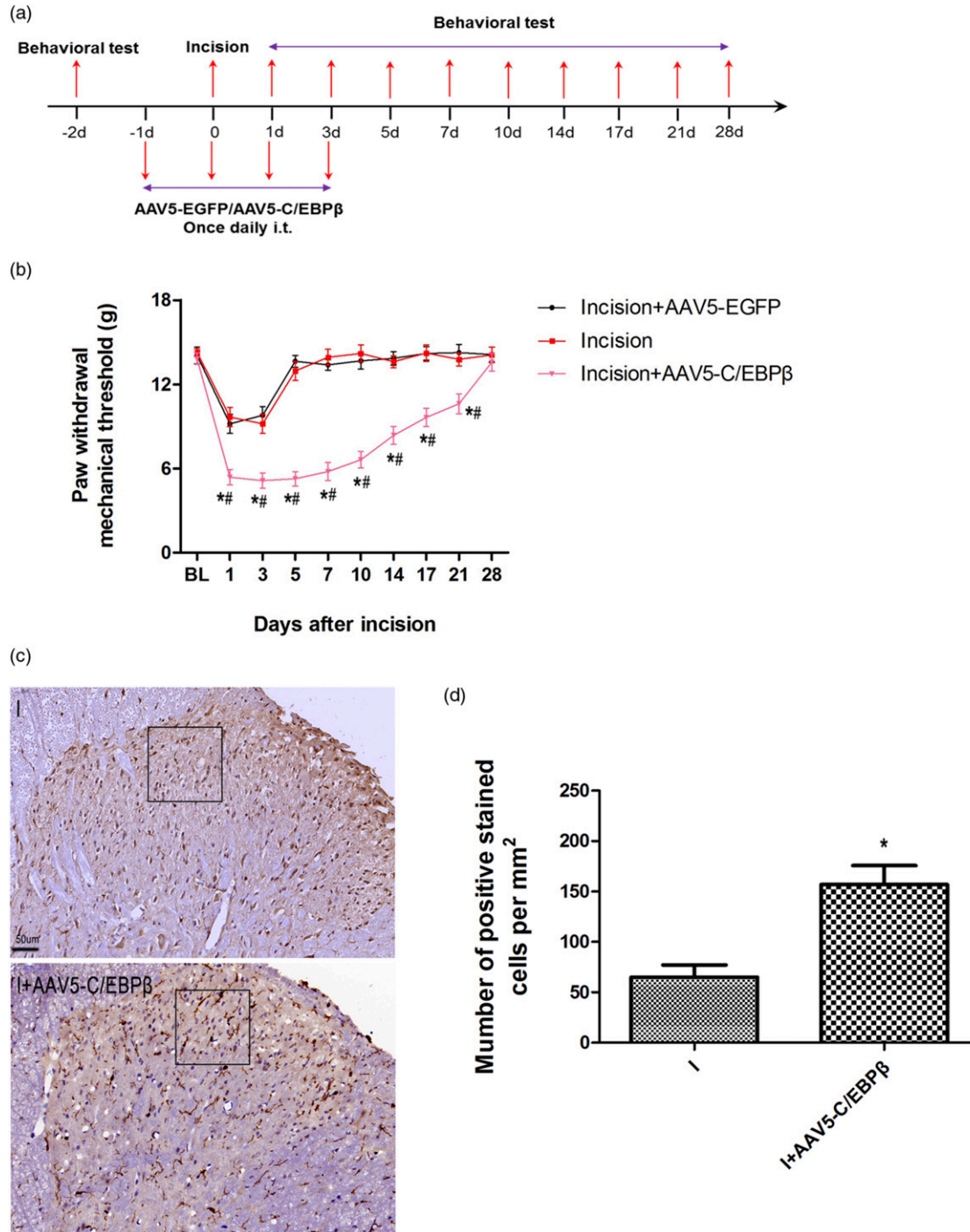
Figure 6. Mimicking the SPS-induced increase in C/EBPβ in the spinal cord prolonged postsurgical pain symptoms and potentiated microglial activation. (a) The experimental design and time schedule. (b) Injection of AAV5-C/EBPβ yielded lower PWMT values and prolonged the duration of postsurgical pain compared with those in other groups (n=6 per group). AAV5-C/EBPβ was intrathecally injected once daily for consecutive 5 days (1 day before incision to 3 days after incision). (c) Immunohistochemical staining for the number of IBA-1-positive cells in spinal cord dorsal horn. (d) Rats injected with AAV5-C/EBPβ and subjected to incisional surgery displayed obvious increases in IBA-1-positive cells in the dorsal horn (n=6 per group). Group Incision: rats underwent an incisional surgery with sham SPS procedure; Group Incision+AAV5-C/EBPβ: rats underwent an incisional surgery and microinjection of AAV5-C/EBPβ once daily for consecutive 5 days; Group Incision+AAV5-EGFP: rats underwent an incisional surgery and microinjection of AAV5-EGFP once daily for consecutive 5 days $*p < 0.05$ compared with Group Incision; $^{\#}p < 0.05$ compared with Group Incision+AAV5-EGFP.
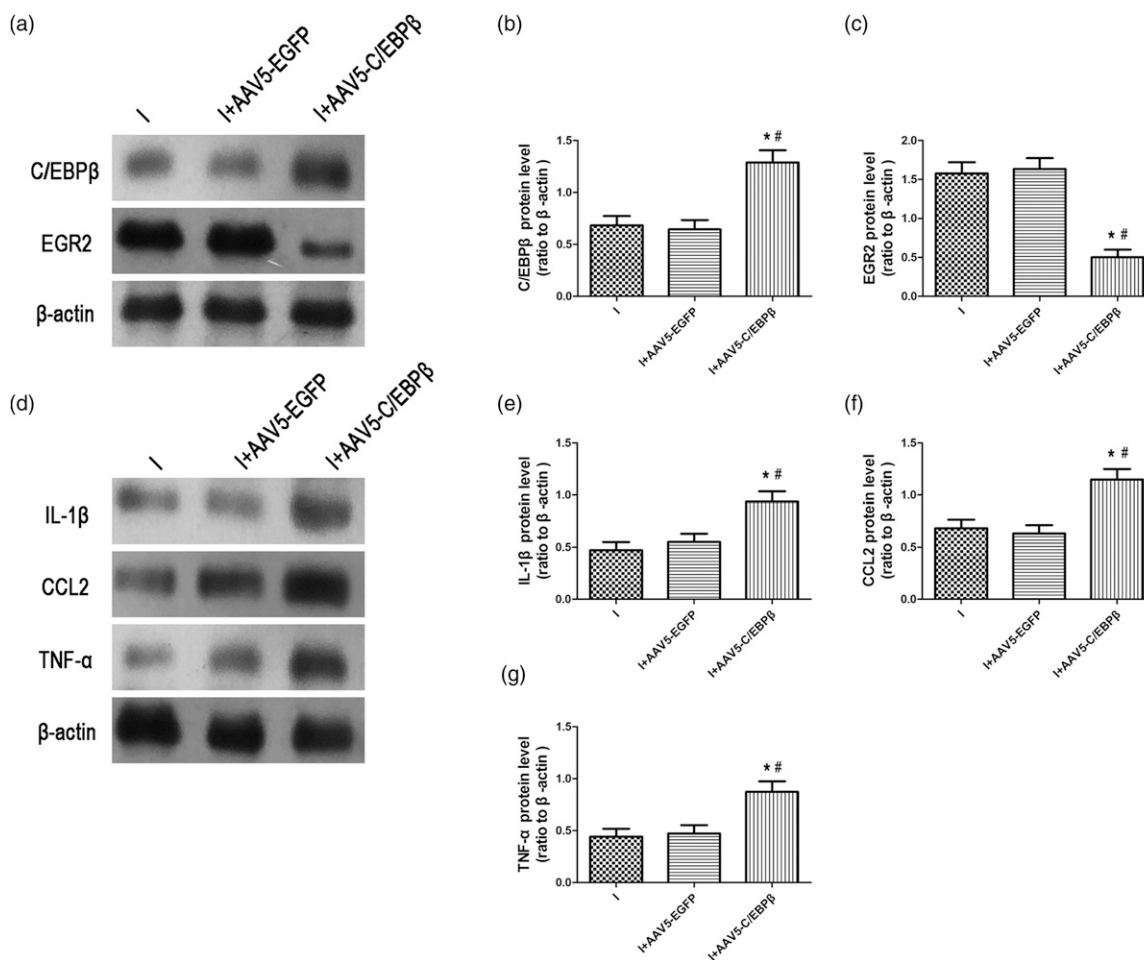
Figure 7. Mimicking the SPS-induced increase in C/EBPβ in the spinal cord potentiated C/EBPβ activation and pro-inflammatory cytokine expression. (a) Western blotting for the expression of C/EBPβ and EGR2 protein in spinal cord dorsal horn. (b–c) Results revealed that pre-injection of AAV5-C/EBPβ significantly increased C/EBPβ protein levels but markedly reduced EGR2 protein levels (n=6 per group). (d) Western blotting for the proinflammatory cytokines expression of IL-1β, CCL2 and TNF-α protein in spinal cord dorsal horn. (e–g) Rats injected with AAV5-C/EBPβ and subjected to incisional surgery showed marked increases in the pro-inflammatory cytokines IL-1β, CCL2, and TNF-α (n=6 per group). Group I: rats underwent an incisional surgery with sham SPS procedure; Group I+AAV5-C/EBPβ: rats underwent an incisional surgery and microinjection of AAV5-C/EBPβ once daily for consecutive 5 days; Group I+AAV5-EGFP: rats underwent an incisional surgery and microinjection of AAV5-EGFP once daily for consecutive 5 days *$p < 0.05$ compared with Group I; #$p < 0.05$ compared with Group I+AAV5-EGFP.

The present study has some limitations. Male rats were used to avoid confounding effects of the estrous cycle of females on our experiments, but the incidence of pre-operative stress is high in female patients.[47] Pre-operative stress induced postoperative hyperalgesia in female rats is mediated by the 5-HT2B receptor in spinal neurons ,[48] rather than spinal microglia. Recent evidence has suggested that microglia has sexually dimorphic role in pain.[49] Therefore, our findings should be verified in female rodents.

In summary, the stress-induced priming of C/EBPβ in spinal microglia initiated the pre-operative stress-induced prolongation of postsurgical pain. Inhibition of spinal C/EBPβ by microinjection of C/EBPβ siRNA promoted recovery from pre-operative SPS-induced prolongation of postsurgical pain by reducing microglial activation and pro-inflammatory cytokine expression. Thus, C/EBPβ may be a therapeutic target for chronic postsurgical pain induced by pre-operative stress.

Declaration of conflicting interests

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Ethical standards

This article does not contain any studies with human participants. The experimental procedures were approved by the Use Committee in Nanjing University and Institutional Animal Care.

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