YY1 promotes SOCS3 expression to inhibit STAT3-mediated neuroinflammation and neuropathic pain

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Abstract. Neuropathic pain is induced by primary injury and dysfunction of the nervous system, and is accompanied by the activation of inflammation signaling pathways. Yin Yang 1 (YY1) is reported to be involved in inflammation; however, its role in the development of neuropathic pain is still unclear. In the present study, a neuropathic pain model was established using the bilateral chronic constriction injury (bCCI) method in rats. The indexes of neuropathic pain were detected, including paw mechanical withdrawal threshold (MWT), paw thermal withdrawal latency (PTWL) and paw frequency in response to cold stimulus, characterizing the symptoms of mechanical allodynia, thermal hyperalgesia and cold hyperalgesia, respectively. YY1 mRNA expression was significantly decreased in the spinal cord cells of bCCI rats. In addition, YY1 was overexpressed in the bCCI rats by intrathecally injecting different doses of the pcDNA-YY1. YY1 reduced rat mechanical allodynia, thermal hyperalgesia and cold hyperalgesia in a dose-dependent manner. Furthermore, YY1 increased the expression of suppressor of cytokine signaling 3 (SOCS3) and suppressed signal transducer and activator of transcription 3 (STAT3)-mediated production of inflammatory factors in a dose-dependent manner. Finally, YY1 were respectively overexpressed and knocked down in primary spinal cord cells. The results revealed that YY1 overexpression promoted SOCS3 expression, increased cell proliferation and suppressed cell apoptosis, and reduced the activation of STAT3 and STAT3-mediated production of inflammatory factors. YY1 knockdown induced the opposite effect to that observed following YY1 overexpression. Furthermore, blockade of SOCS3 by SOCS3-antibody abrogated the effect of YY1 overexpression on the suppression of SOCS3-mediated STAT3 activation and inflammation. In conclusion, YY1 alleviated neuropathic pain by inhibiting the STAT3 signaling pathway, which may be due to the upregulation of SOCS3 expression.

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Introduction

Chronic neuropathic pain is a chronic and highly incapacitating complex disease, which is aroused by primary injury and dysfunction of the nervous system, accompanied by activation of inflammation signaling pathways (1). Neuropathic pain may result from many factors including physical injury, metabolic or nutritional nerve change, virus infection, neurotoxicity of drugs or radiotherapy, ischemic nerve damage, neurotransmitter dysfunction, etc. Individuals with neuropathic pain displayed high sensitivity to mechanical, heat and cold stimulation, and local and/or systemic inflammation reaction. In order to develop novel therapeutic target or strategy for neuropathic, it is essential to better understand its underlying mechanisms. The signal transducer and activator of transcription 3 (STAT3) pathway is a key pathway mediating inflammation, which plays an important role in synaptic plasticity, neural degeneration and memory formation in central nervous system. Recently, it has been revealed that activation of STAT3 was important to the development of chronic pain, and inhibition of STAT3 activation has been regarded as a promising therapy for neuropathic pain.

The suppressor of cytokine signaling (SOCS) proteins have previously been reported to negatively regulate the STAT3 signaling, of which SOCS3 are the most effective member (2). SOCS3 can inhibit STAT3 activation and blocks STAT3 signaling, thereby limiting some of the pathophysiological consequences (3,4). Therefore, SOCS3 is a potential target for the effective treatment of neuropathic pain.

Yin Yang 1 (YY1) is a commonly expressed zinc-finger DNA-binding transcription factor in a variety of cells, which can interact with other transcription factors (5). It is reported that YY1 improved tumor cells growth and suppressed apoptosis through negative regulation of TP53 (6). YY1 is also involved in airway inflammation, and is a positive regulator of

many inflammatory cytokines in T cells, including IL-4, IL-5 and IL-13 (7). However, the function of YY1 in neuropathic pain is still unclear. In this study, we detected the YY1 level in neuropathic pain model of rats and analyzed its influence on neuropathic pain. Then, we further explored weather the effect of YY1 on pain is involved in regulation of the SOCS3/STAT3 signaling pathway.

Materials and methods

Animals. Adult female Sprague-Dawley rats (195-215 g) were purchased from the Experimental Animal Centre of Shandong University (China). Animals were housed in individual cages at 23.0 ± 1˚C under a 12 h light/dark cycle and were given free access to water and food. All animal protocols were conducted in accordance with the Institutional Animal Care and Use of the Yantai Yuhuangding Hospital (Shandong, China). The present study was approved by the Ethics Committee of Yantai Yuhuangding Hospital.

Neuropathic pain model. Bilateral Chronic constriction injury (bCCI) of rats sciatic nerve was a common experimental model for neuropathic pain (8). The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The mid-thigh on both side was incised and the heads of the biceps femoris muscle was separated to expose the sciatic nerve. 4-0 chromic gut was carried out ligate the sciatic nerve at four sites. The wound was closed with absorbable sutures. Sham-operated rats received a similar exposure but without nerve isolation and ligation. The rats were closely monitored following surgery. Naïve group did not receive any surgery.

Mechanical allodynia. Rats were acclimatized in suspended cages. The hind paws were probed with calibrated Electronic von Frey device (IITC Life) erected to the plantar surface and kept for ~5 sec. A positive response represented a sharp withdrawal of the paw. The mechanical withdrawal threshold (MWT) was indicated as the average of the measurements in each test session.

Thermal hyperalgesia. Thermal hyperalgesia was detected with a Plantar Test Apparatus for Mice and Rats (IITC). Rats were acclimated on a glass floor above a radiant heat producer that was aimed at the plantar surface of the hind paw. In each test session, triple measurements of latency were done for each hind paw. Each test was done with intervals greater than 3 min and the device was cutoff for 30 sec in the intervals of each test to avoid tissue damage. The thermal withdrawal latency (TWL) was indicated as the average of the measurements in each test session.

Cold hyperalgesia. A drop of acetone was gently applied to each hind paw at room temperature. A rapid hind paw withdrawal in response to the volatilization of acetone was considered as a sign of cold allodynia. The test was repeated 3 times for every hind paw, with an interval of 3 min. Times of shaking, lifting or licking the paw during 1 min were recorded. An increase in the rate of withdrawal response interpreted as increased cold sensitivity.

Culture of primary spinal cord neurons and cell transfection. A total of 6 SD rats (3 months old) were anesthetized intraperitoneal injection with 3% pentobarbital sodium at a dose of 30 mg/kg body weight. Spinal cord was isolated from L4-L6 of the rats after sacrificed by cervical dislocation and digested with 0.25% trypsin for 30 min followed by 0.2% collagenase II for 2 h. The cells were washed with D-Hank's buffer (Invitrogen; Thermo Fisher Scientific, Inc.) for 3 times and then seeded in the plates with DMEM/F12 (containing 4.5 g/l glucose) supplemented with 20% FBS and 4 mM L-glutamine. Cells were cultured in a 37˚C humidified cell culture chamber with 5% CO_2.

On reaching 70% confluence, 2 μg/ml pcDNA-YY1 (constructed by GenScript Company, Nanjing, China) and 50 nM YY1 siRNA (designed and synthesized by Ribobio Technology, Guangzhou, China) were respectively transfected in to the cells with Lipofectamine® 3000 reagent (Thermo Fisher Scientific, Inc.) in accordance with the manufacturers' instructions. After 48 h, the cells were harvested.

Cell viability assay. Cell viability was performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Amresco, Solon, OH, USA) (9). After transfection for 48 h, cells were plated in 96-well plates (Corning, MA, USA) at 1.0 x 10^4 cells per well and incubated for 12 h. MTT solution (20 μl/well, 5 mg/ml) were added to the well and incubated for 4 h. The media were removed and 200 μl DMSO per well was added to dissolve formazan crystals. Finally, the absorbance was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 570 nm wavelength.

Apoptosis assay. In 60 mm plates, cells were collected by centrifugation after transfection for 72 h. the Apoptosis Assay was performed using Apoptosis Detection kit II (BD Biosciences, San Diego, CA, USA). Cells were resuspended in 500 μl of 1X Binding Buffer with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) and incubated at room temperature for 15 min in the dark. The flow cytometry (BD Biosciences) was used for the evaluation of percentage of apoptotic cells. The data was from typically 10,000 cells and analyzed in Cell Quest software.

Fluorometry assay. Intracellular reactive oxygen species. (ROS) generation was evaluated using a fluorometry assay (10). Cell was collected with trypsin and centrifuged. Cell pellets were exposed to dichlorodihydrofluorescein diacetate (DCFH-DA) (1:1,000; Sigma, USA) for 20 min at 37°C in the darkroom. The flow cytometry (BD Biosciences) was used to measure the ROS level, which was the mean of the fluorescence for each treatment with typically 10,000 cells analyzed. All experiments were conducted for three times.

Protein estimation by ELISA. Total protein concentration of samples was determined with a Bicinchoninic Acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) IL-1β and IL-6 level were determined with ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). Sample optical densities were measured at 450 nm with a reference filter set at 570 nm.

Table I. Primers applied in reverse transcription-quantitative polymerase chain reaction analysis.

Target	Sequences (5'-3')	Product size (bp)
YY1	F: GCCCTCATAAAGGCTGCACAAAGAT	223
	R: GTGCGCAAATTGAAGTCCAGTGAA	
SOCS3	F: TCACGGCTGCCAACATCTGG	228
	R: CGGCGGCGGGAAACTTG	
COX2	F: TCTTTGCCCAGCACTTCACTCA	367
	R: TCAGGATGCTCCTGTTTGA	
CCL2	F: ATGCAGTTAATGCCCCACTC	167
	R: TTCCTTATTGGGGTCAGCAC	
IL-1β	F: CACCTTCTTTTCCTTCATCTT	238
	R: GTCGTTGCTTGTCTCTCCTTGTA	
IL-6	F: AAGTTTCTCTCCGCAAGAGACTTCCAG	326
	R: AGGCAAATTTCCTGGTTATATCCAGTT	
TNF-α	F: GTAGCCCACGTCGTAGCAAAC	196
	R: TGTGGGTGAGGAGCACATAGTC	
GAPDH	F: ATCCCATCACCATCTTCCAG	322
	R: CCATCACGCCACAGTTTCC	

F, forward; R, reverse; YY1, Yin Yang 1; SOCS3, suppressor of cytokine signaling 3; COX2, cyclooxygenase 2; CCL2, chemokine (C-C motif) ligand 2; IL, interleukin; TNF, tumor necrosis factor.

Cytokine concentrations were calculated according to the standard curves.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration of total RNA was determined using a NanoDrop 2000c (Thermo Fisher Scientific, Inc.). 1 μg of RNA was used to synthesize cDNA with the PrimeScript™ RT master mix (Takara, Japan). RT-qPCR was performed using SYBR Premix Ex Taq (Takara, Japan) and the thermocycling conditions were as followed: 42˚C for 30 min, then 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and 60˚C for 1 min. The relative amount of transcript was quantified via the comparative threshold cycle method using GAPDH as the endogenous reference. Transcript levels in each rat were determined with the formula $2^{-\Delta\Delta Cq}$ (11). The primers for amplifying all the genes were selected from reported references and are presented in Table I.

Western blot analysis. Protein concentration was determined with a BCA protein assay kit (Pierce). Extracted proteins were separated by SDS-PAGE, transferred on a nitrocellulose membrane (NC; Millipore, USA). Anti-YY1 (AV100899; Sigma, USA), anti-SOCS3 (sc-9023; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-STAT3 (sc-482; Santa Cruz Biotechnology, Inc.), anti-phosphorylated STAT3 (sc-135649; Santa Cruz Biotechnology, Inc.), anti-CCL2 (66272-2-lg; ProteinTech, USA), anti-COX2 (SAB5500087; Sigma, USA), anti-GCR (ab2768; Abcam, UK) and anti-GAPDH (sc-25778; Santa Cruz Biotechnology, Inc.) antibodies were used to block the membrane overnight at 4°C. After washing with TBST for 3 times, the membrane was incubated with horseradish peroxidase conjugated antibodies (1:2,000; bs-0295-HRP; Bioss, China). Then the membrane was incubated with western chemiluminescent HRP substrate (Millipore, USA) to detect the signal. Image J was used for statistical analysis. Three consistent independent experiments were performed to analyze the results.

Statistical analysis. All data is presented as the mean ± standard deviation. Two factors (group and times) repeated measures analysis of variance (ANOVA) was used to analyze the time course of bCCI-induced tactile allodynia between groups. Multiple comparisons were analyzed with ANOVA and Dunnett's post hoc test. Comparisons between two groups were analyzed by the Student's t-test. Data were processed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

YY1 was decreased in rats with bCCI. To investigate the involvement of YY1 in neuropathic pain, the bCCI rats were established. Before and after surgery, the recorded scores exhibited no significant differences between left and right hind paws from the stimulus at any of the time, the mean scores of left and right hind paws were used to analyze the results. At days 0, 3, 7, 14 and 21, the indexes of neuropathic pain including MWT, paw thermal withdrawal latency (PTWL) and paw frequency in response to cold stimulus were detected in each group, respectively characterizing the mechanical allodynia, thermal hyperalgesia and cold hyperalgesia of the rats. Compared with sham group, MWT and PTWL of bCCI group were significantly decreased from 3 days to

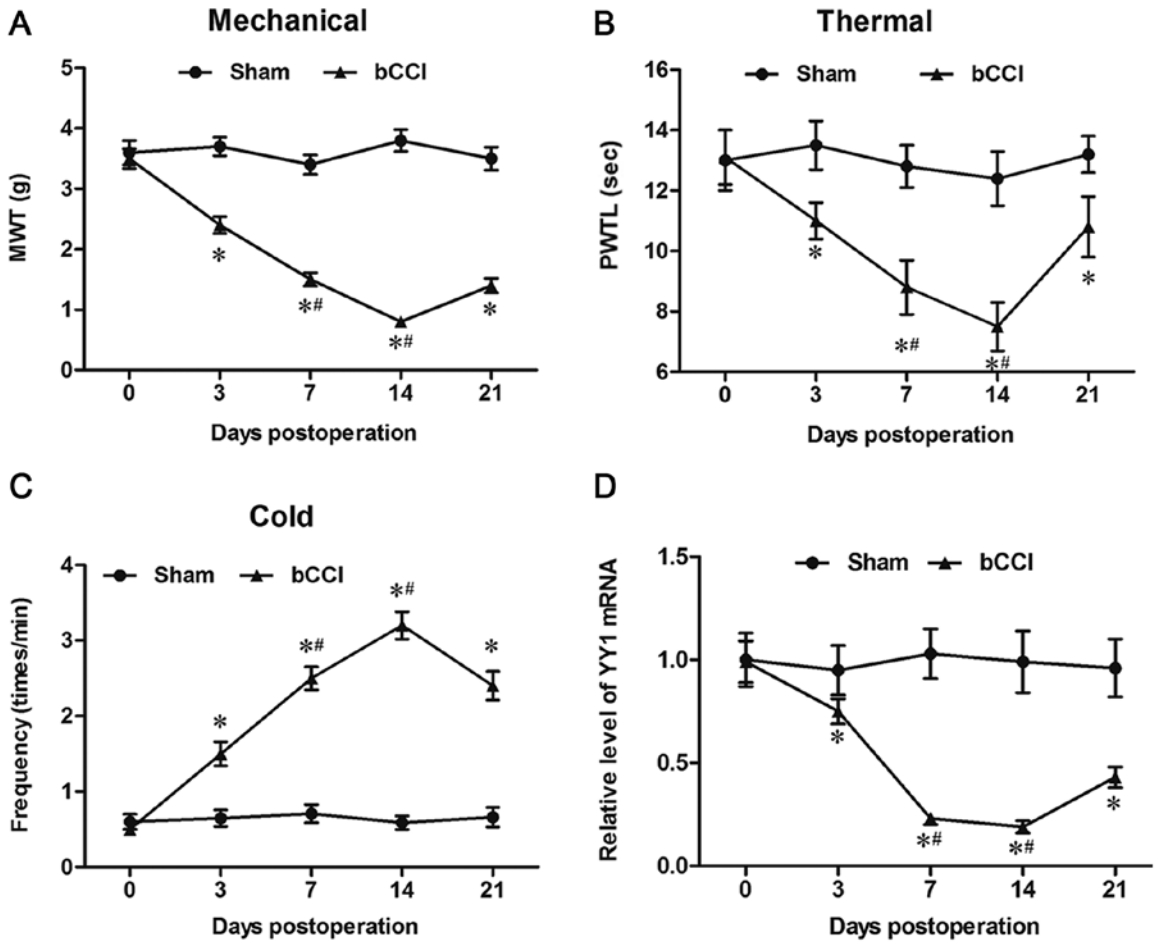

Figure 1. YY1 is downregulated in the spinal cord cells of bCCI rats. A neuropathic pain rat model was established using the bCCI method. On days 0, 3, 7, 14 and 21, the indexes of neuropathic pain including (A) MWT, (B) PTWL and (C) paw frequency were detected in response to a cold stimulus in each group, characterizing the mechanical allodynia, thermal hyperalgesia and cold hyperalgesia of the rats. (D) In addition, spinal cord cells were isolated from the L4-L6 of the rats, and the expression of YY1 was detected by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean ± standard deviation (n=6). *P<0.05 vs. Sham group; #P<0.05 vs. day 3 of the bCCI group. YY1, Yin Yang 1; bCCI, bilateral chronic constriction injury; MWT, mechanical withdrawal threshold; PTWL, paw thermal withdrawal latency.

21 days after operation and reached its lowest point on day 14 (Fig. 1A and B). From 3 days after surgery, the times of the responses of bCCI rats to cold acetone was significantly higher than that in sham group and reached its highest point on day 14 (Fig. 1C). Then we determined the expression of YY1 in bCCI rats. The results suggested that YY1 mRNA level was significantly decreased in rats with bCCI compared to the sham group (Fig. 1D).

YY1 reduced neuropathic pain symptoms of bCCI rats. Different concentrations of pcDNA-YY1 expression vectors were injected intrathecally, and expression of YY1 mRNA and protein was detected with qPCR and Western blotting. The results showed that, compared with the Vector group, 4 mg/kg pcDNA-YY1 transfection significantly increased and 8 mg/kg pcDNA-YY1 further increased the level of YY1 mRNA (Fig. 2A). Moreover, in pcDNA-YY1 overexpression groups (4 mg/kg and 8 mg/kg groups), YY1 mRNA displayed a robust increase at day 3 and a drop at days 7 and 14 (Fig. 2A). YY1 protein was being increased from day 0 to day 14 in the 8 mg/kg pcDNA-YY1 group, compared with the Vector group (Fig. 2B). The values of MWT and PMWT of bCCI group were markedly increased by treatment with 4 mg/kg pcDNA-YY1 vector, and further increased by treatment with 8 mg/kg (Fig. 2C and D). Consistently, the paw frequency in response to cold stimulus of bCCI group was significantly lowered by treatment with 4 mg/kg pcDNA-YY1 vector, and further lowered by treatment with 8 mg/kg (Fig. 2E). As a key signaling pathway regulating neuropathic pain, activation of STAT3 was obviously suppressed by overexpression of YY1, and expression of SOCS3, a negative regulator of STAT3 was obviously promoted (Fig. 2F).

YY1 significantly inhibited inflammation and ROS overproduction in bCCI rats. Neuroinflammation and oxidative stress are direct pathological mechanism of neuropathic pain. We checked the contents of ROS and inflammatory cytokines including IL-1β, IL-6 and TNF-α in the spinal cord cells from each group. The results from qPCR showed that the expression levels of IL-1β (Fig. 3A), IL-6 (Fig. 3B), and TNF-α (Fig. 3C) were significantly decreased by treatment with 4 mg/kg pcDNA-YY1, and further decreased by treatment with 8 mg/kg pcDNA-YY1. Moreover, YY1 decreased the ROS content in bCCI rats in a dose-dependent manner (Fig. 3D). These data indicated that overexpression of YY1 inhibited neuroinflammation and oxidative stress in the spinal cord cells bCCI rats.

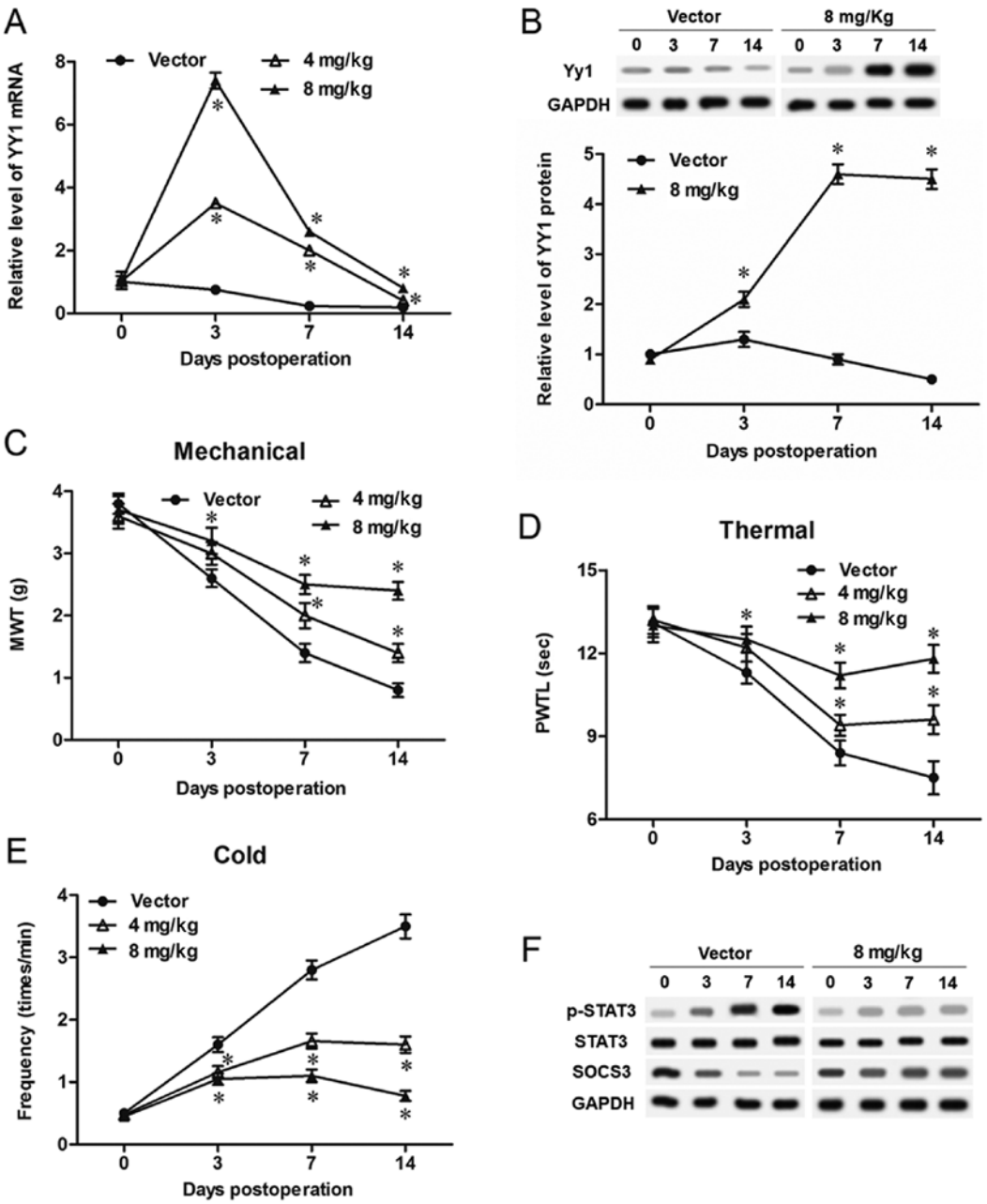

Figure 2. YY1 relieves neuropathic pain symptoms in bCCI rats. YY1 was overexpressed in bCCI rats by intrathecally injecting different doses (4 or 8 mg/kg) of the pcDNA-YY1 expression vector. The levels of YY1 (A) mRNA and (B) protein in spinal cord cells were detected on days 0, 3, 7 and 14. The effect of YY1 overexpression on the indexes of neuropathic pain including (C) MWT, (D) PMWT and (E) paw frequency in response to cold stimuli were detected on days 0, 3, 7 and 14. (F) YY1 promoted SOCS3 protein expression and suppressed the activation of STAT3. Data are expressed as the mean ± standard deviation (n=6). *P<0.05 vs. Vector. YY1, Yin Yang 1; bCCI, bilateral chronic constriction injury; MWT, mechanical withdrawal threshold; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3.

YY1 inhibited STAT3 signaling activation through upregulating SOCS3 expression. To verify the role of YY1 in STAT3 signaling pathway, primary spinal cord cells were cultured, and expression of YY1 was manipulated in the cells by transfection with pcDNA-YY1 or YY1 siRNA. After 72 h, we detected the changes in the levels of its downstream gene glucocorticoid receptor (GCR), SOCS3, phosphorylated STAT3 (p STAT3) and downstream target genes of STAT3 including cyclooxygenase 2 (COX2) and chemokine (C-C motif) ligand 2 (CCL2). The levels of YY1 mRNA and protein were significantly increased by pcDNA-YY1 and decreased by YY1 siRNA (Fig. 4A and E). In response to YY1 overexpression, expression of SOCS3 and GCR was significantly promoted, while the levels of COX2, CCL2 and pSTAT3 were significantly decreased (Fig. 4B-E). In contrast, YY1 knockdown suppressed expression of SOCS3 and glucocorticoid receptor, and increased the levels of COX2, CCL2 and phosphorylated STAT3 (Fig. 4B-E). To validate that YY1 deregulated the activation of the STAT3 pathway through upregulation of SOCS3, SOCS3 antibody was used to neutralizing SOCS3 when YY1 was overexpressed. Our results showed that blockade of SOCS3 abrogated the effect of YY1 overexpression on the upregulation of COX2, CCL2 and pSTAT3 (Fig. 4B-E). These results indicated that YY1

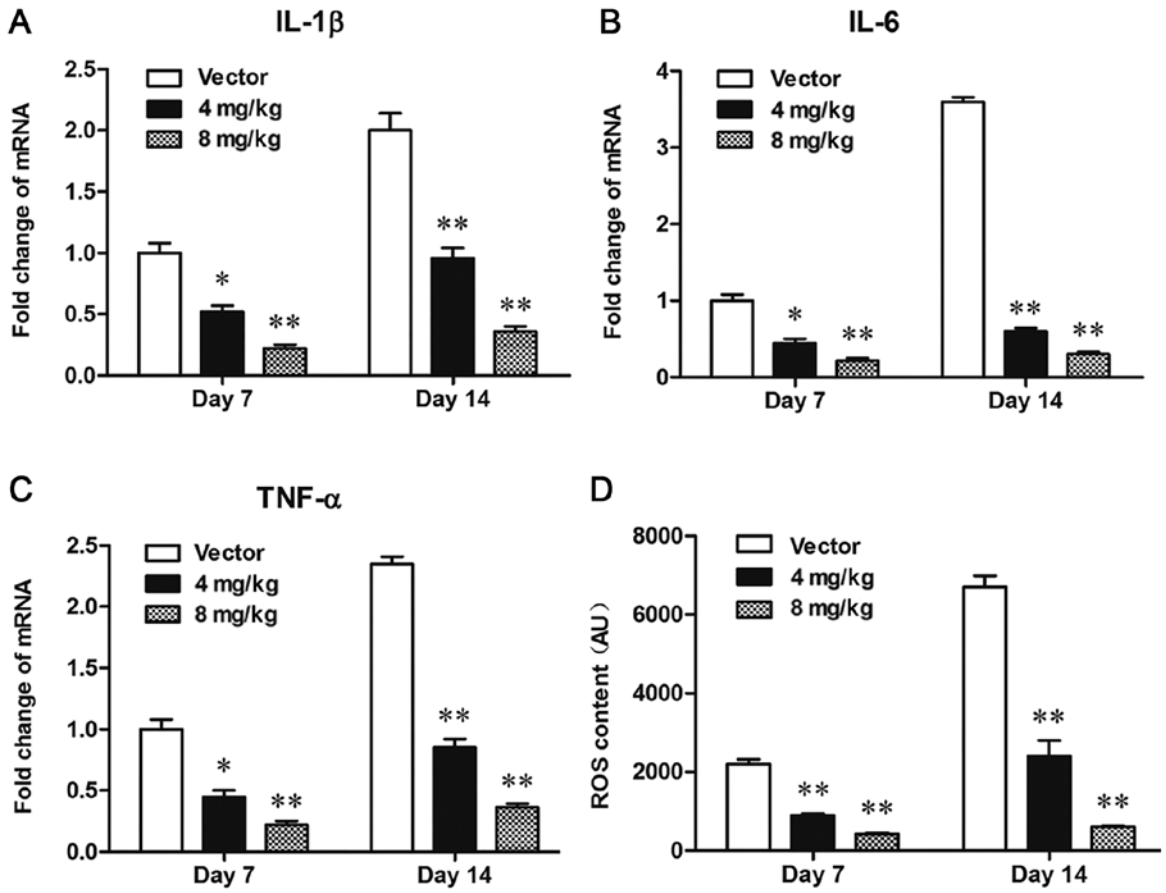

Figure 3. YY1 overexpression suppresses neuroinflammation and ROS production in bCCI rats. YY1 overexpression suppressed the mRNA levels of pro-inflammatory genes in spinal cord cells of bCCI rats, including (A) IL-1β, (B) IL-6 and (C) TNF-α. (D) YY1 overexpression suppressed ROS content in the spinal cord cells of bCCI rats. YY1 was overexpressed in bCCI rats by intrathecally injecting different doses (4 or 8 mg/kg) of the pcDNA-YY1 expression vector. The mRNA levels of IL-1β, IL-6 and TNF-α were detected by reverse transcription-quantitative polymerase chain reaction on days 7 and 14 in spinal cord cells. ROS content was detected using the dichlorodihydrofluorescein diacetate fluorescence method. Data are expressed as the mean ± standard deviation (n=6). *P<0.05 and **P<0.01 vs. Vector. TNF, tumor necrosis factor; YY1, Yin Yang 1; bCCI, bilateral chronic constriction injury; ROS, reactive oxygen species; IL, interleukin; AU, arbitrary units.

inhibited STAT3 signaling activation through upregulating the expression of SOCS3.

YY1 improved cell viability, and suppressed cell apoptosis and secretion of inflammatory factors. Finally, the effect of YY1 on cell survival and inflammation in the spinal cord cells was investigated. Our results from MTT, flow cytometry and ELISA indicated that overexpression of YY1 increased cell viability, inhibited apoptosis, and reduced the secretion of inflammatory factors IL-1β and IL-6, whereas YY1 knockdown displayed an opposite effect (Fig. 5A-D). Moreover, blockade of SOCS3 abrogated the effect of YY1 overexpression on cell viability, cell apoptosis and inflammation (Fig. 5A-D).

Discussion

As a poorly managed clinical problem, neuropathic pain may also result from immune activation and proinflammatory cytokine release (11). Transcription factors act as central regulators of the immune response to stimuli (12). YY1 is a commonly expressed zinc-finger DNA-binding transcription factor in a variety of cells, which can interact with other transcription factors (5). We all know that YY1 is a 'versatile' regulator in gene expression and pathogenesis of various diseases. For example, YY1 may function as a tumor suppressor or a tumor promoting gene in different types of cancer. In this study, a neuropathic pain rat model with the bCCI method was established, and we found that YY1 was downregulated in bCCI neuropathic pain model and its overexpression attenuated neuropathic pain symptoms of bCCI rats. We showed that YY1 can suppress the activation of STAT3, which is regarded as a key contributor to neuropathic pain. What is more, upregulation of YY1 significantly inhibited the production of pro-inflammation factors IL-1β, TNF-α and IL-6 in bCCI rats. These results implicated that YY1 attenuate neuropathic pain symptoms via suppression of the STAT3 pathway. Most of existing papers showed that YY1 had pro-inflammatory effects. However, a few of studies demonstrated that YY1 could suppress expression of pro-inflammatory genes and functioned as an anti-inflammatory gene (13-16). For example, YY1 was shown to strongly repress transcription of the proinflammatory gene matrix metalloproteinase-9 in brain neurons (15). Furthermore, in many other types of cells, YY1 was shown to upregulate glucocorticoid receptor, the which has been regarded as a therapeutic target for chronic pains (17-20). In this study, we found that glucocorticoid receptor was also upregulated by YY1 overexpression in spinal cord neurons, which may partially

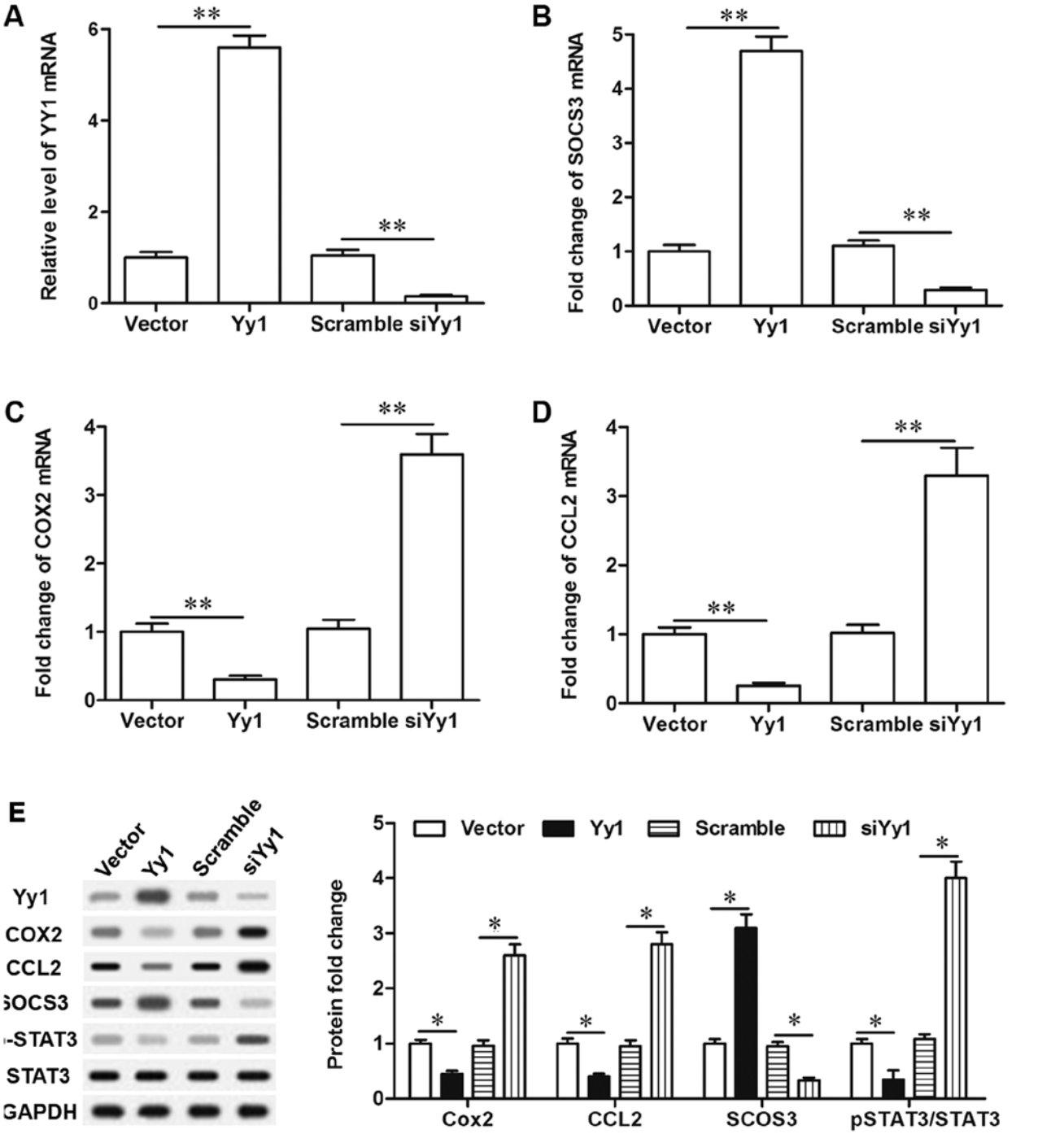

Figure 4. YY1 negatively regulates the activation of the STAT3 signaling pathway via upregulation of SOCS3. (A) The overexpression and knockdown efficiencies of pcDNA-YY1 and YY1 siRNA. (B) YY1 positively regulated the expression of SOCS3. (C) YY1 negatively regulated the expression of Cox-2. (D) YY1 negatively regulated the expression of CCL2. (E) YY1 negatively regulated the activation of the STAT3 signaling pathway via upregulation of SOCS3. Primary spinal cord cells were isolated from young rats. Upon reaching ~70% confluence, the cells were treated with 2 μg/ml pcDNA empty vector, 2 μg/ml pcDNA-YY1, 50 nM scrambled siRNA, 50 nM YY1 siRNA, or 2 μg/ml pcDNA-YY1 plus 5 μg/ml SOCS3 neutralizing antibody. Following incubation for 72 h, total RNA and total protein were extracted from the cells. The mRNA levels of YY1, SOCS3, Cox-2 and CCL2 were detected with reverse transcription-quantitative polymerase chain reaction, and the protein levels of YY1, GCR, SOCS3, Cox-2, CCL2, STAT3 and pSTAT3 were detected with western blotting. Data are expressed as the mean ± standard deviation (n=4). *P<0.05 and **P<0.01. YY1, Yin Yang 1; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; GCR, glucocorticoid receptor; COX2, cyclooxygenase 2; CCL2, chemokine (C-C motif) ligand 2; p-, phosphorylated.

contribute to the anti-neuroinflammation and neuropathic pain alleviation of YY1, apart from upregulation of SOCS3.

In the overexpression experiments *in vivo*, 4 mg/kg and 8 mg/kg pcDNA-YY1 transfection significantly increased the level of YY1 mRNA, compared with the control. Moreover, in pcDNA-YY1 overexpression groups, YY1 mRNA displayed a robust increase at day 3 and a drop at days 7 and 14. It is worth mentioning that, unlike the change of YY1 mRNA expression, YY1 protein expression was being increased from day 0 to day 14 in the 8 mg/kg pcDNA-YY1 group. We carefully checked

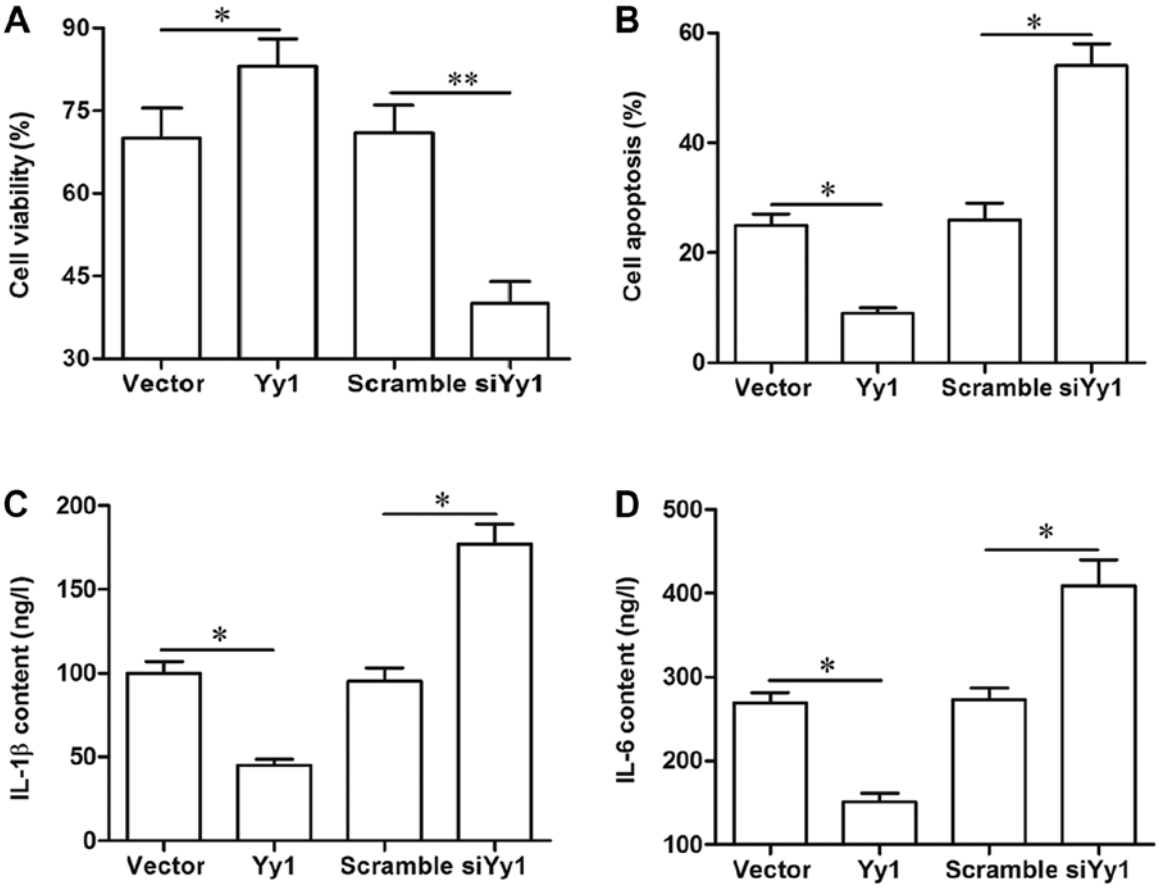

Figure 5. YY1 improves cell survival and suppresses inflammation. (A) YY1 could improve the viability of spinal cord cells, which was antagonized by the blockade of SOCS3. (B) YY1 negatively regulated the apoptosis of spinal cord cells, which was antagonized by the blockade of SOCS3. YY1 negatively regulated the secretion of (C) IL-6 and (D) IL-1β. Primary spinal cord cells were isolated from young rats. Upon reaching ~70% confluence, the cells were treated with 2 μg/ml pcDNA empty vector, 2 μg/ml pcDNA-YY1, 50 nM scrambled siRNA, 50 nM YY1 siRNA, or 2 μg/ml pcDNA-YY1 plus 5 μg/ml SOCS3 neutralizing antibody. Following incubation for 72 h, cell viability was detected via the MTT method, cell apoptosis was determined by flow cytometry, and the secretion of IL-6 and IL-1β was evaluated with ELISA in the supernatant of the spinal cord cells. Data are expressed as the mean ± standard deviation (n=4). *P<0.05 and **P<0.01. YY1, Yin Yang 1; SOCS3, suppressor of cytokine signaling 3; IL, interleukin; si-/siRNA, small interfering RNA; n.s., not significant.

these results and found that the expression patterns of YY1 mRNA and its protein indeed are not consistent, which is probably because the YY1 protein expression is lagged behind the expression of YY1 mRNA. In fact, it is not uncommon that the expression patterns of mRNA and protein of a gene are not consistent. For example, during porcine adipogenesis, levels of the transcription factor PU.1 mRNA increased at day 2 and then gradually decreased, but it is interestingly that PU.1 protein level was being increased during differentiation (21).

Spinal cord neurons play important roles in the occurrence and maintenance of neuropathic pain during nerve injury and systematic inflammation (22). Neuropathic pain, embodied as pain hypersensitivity, was thought to be mainly resulted from altered neuronal activity in primary sensory and spinal cord neurons. Improvement of spinal cord neuron viability and activity has been used to manage pains (23). In the present study, our results demonstrated YY1 improved the viability and inhibited apoptosis of spinal cord neurons, which explained why YY1 could alleviate neuropathic pain in bCCI rats at the cellular level to some extent. There were rarely reports on the role of YY1 in the behaviors of spinal cord neuron, but its promotion on cell proliferation and inhibition on cell apoptosis have been reported. For example, during skeletal muscle development and regeneration, YY1 was found to be upregulated, and downregulation of YY1 expression had an effect on suppressing proliferation of myoblasts (24). YY1 was also proven to promote the viability of many other types of cells, including many types of cancer cells and leukomonocytes (25-27). As for the role of YY1 in apoptosis, it was found to be one of only a few transcription factors targeted by caspases (28). During many pathological processes, YY1 was upregulated or activated in response to cell stress to block cell death signaling pathways (29-31).

The STAT signaling is a key pathway activated by nerve injury and plays an important role in nerve survival and regeneration (32). STAT3 was reported to promote cell damage in the immune response and is activated by phosphorylation (33). Persistent activated STAT3 leads to occurrence of many diseases, including neurological impairment (34). The activation of the STAT3 pathway is also observed in bCCI rats, during which STAT3 was highly phosphorylated (8). Activated STAT3 upregulated downstream inflammation biomarkers COX-2 and CCL2, induced production and release of pro-inflammatory cytokines IL-1, IL-6 and TNF-α, and then resulted in intense inflammatory response and then causing histiocytic death, tissue dysfunction and pain (35-38).

SOCS3 was reported as the most effective negative regulator of the Janus Kinase2/STAT3 signaling (39). Some recent studies indicated that SOCS3 has been identified as a therapeutic target for neuropathic pain (39-41). For example, downregulation of microRNA-218 caused increase in SOCS3 expression and can relieve neuropathic pain in bCCI rats (39). Here, our results showed that upregulation of SOCS3 by YY1 overexpression decreased had a suppression effect on STAT3 activation in spinal cord cells and could relieve neuropathic pain bCCI rats. In conclusion, YY1 is downregulated in bCCI rats, and it upregulates SOCS3 expression to inhibit STAT3-mediated neuroinflammation and neuropathic pain.

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Availability of data and materials

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

Authors' contributions

MYS and KZL designed the present study. MYS, YS and JFM performed the experiments, analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Yantai Yuhuangding Hospital (Shandong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Neville A, Peleg R, Singer Y, Sherf M and Shvartzman P: Chronic pain: A population-based study. Isr Med Assoc J 10: 676-680, 2008.
2. Yoshimura A, Naka T and Kubo M: SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 7: 454-465, 2007.
3. Recio C, Oguiza A, Mallavia B, Lazaro I, Ortiz-Muñoz G, Lopez-Franco O, Egido J and Gomez-Guerrero C: Gene delivery of suppressors of cytokine signaling (SOCS) inhibits inflammation and atherosclerosis development in mice. Basic Res Cardiol 110: 8, 2015.
4. Jo D, Liu D, Yao S, Collins RD and Hawiger J: Intracellular protein therapy with SOCS3 inhibits inflammation and apoptosis. Nat Med 11: 892-898, 2005.
5. Shi Y, Lee JS and Galvin KM: Everything you have ever wanted to know about Yin Yang 1..... Biochim Biophys Acta 1332: F49-F66, 1997.
6. Sui G, Affar el B, Shi Y, Brignone C, Wall NR, Yin P, Donohoe M, Luke MP, Calvo D, Grossman SR and Shi Y: Yin yang 1 is a negative regulator of p53. Cell 117: 859-872, 2004.
7. Guo J, Lin X, Williams MA, Hamid Q and Georas SN: Yin-yang 1 regulates effector cytokine gene expression and T(H)2 immune responses. J Allergy Clin Immunol 122: 195-201, 201.e1-e5, 2008.
8. Xue ZJ, Shen L, Wang ZY, Hui SY, Huang YG and Ma C: STAT3 inhibitor WP1066 as a novel therapeutic agent for bCCI neuropathic pain rats. Brain Res 1583: 79-88, 2014.
9. Soares Nda C, Teodoro AJ, Oliveira FL, Santos CA, Takiya CM, Junior OS, Bianco M, Junior AP, Nasciutti LE, Ferreira LB, *et al*: Influence of lycopene on cell viability cell cycle and apoptosis of human prostate cancer and benign hyperplastic cells. Nutr Cancer 65: 1076-1085, 2013.
10. Sánchez-Fidalgo S, da Silva MS, Cárdeno A, Aparicio-Soto M, Salvador MJ, Frankland Sawaya AC, Souza-Brito AR and de la Lastra CA: Abarema cochliacarpos reduces LPS-induced inflammatory response in murine peritoneal macrophages regulating ROS-MAPK signal pathway. J Ethnopharmacol 149: 140-147, 2013.
11. Watkins LR and Maier SF: Immune regulation of central nervous system functions: From sickness responses to pathological pain. J Intern Med 257: 139-155, 2005.
12. Seiler F, Herr C, Lepper PM, Bals R and Beisswenger C: FOXO transcription factors regulate innate immune mechanisms in respiratory epithelial cells during bacterial infection. In: American thoracic society 2012 international conference may 18-23, 2012 • San Francisco, California, ppA4258, 2012.
13. Lu SY, Rodriguez M and Liao WS: YY1 represses rat serum amyloid A1 gene transcription and is antagonized by NF-kappa B during acute-phase response. Mol Cell Biol 14: 6253-6263, 1994.
14. Yan X, Pan J, Xiao W, Cheng M, Sun Y, Zhang S and Chen Y: Yin Yang 1 (YY1) synergizes with Smad7 to inhibit TGF-β signaling in the nucleus. Sci China Life Sci 57: 128-136, 2014.
15. Rylski M, Amborska R, Zybura K, Mioduszewska B, Michaluk P, Jaworski J and Kaczmarek L: Yin yang 1 is a critical repressor of matrix metalloproteinase-9 expression in brain neurons. J Biol Chem 283: 35140-35153, 2008.
16. Yeh TS, Lin YM, Hsieh RH and Tseng MJ: Association of transcription factor YY1 with the high molecular weight notch complex suppresses the transactivation activity of notch. J Biol Chem 278: 41963-41969, 2003.
17. Takasaki I, Kurihara T, Saegusa H, Zong S and Tanabe T: Effects of glucocorticoid receptor antagonists on allodynia and hyperalgesia in mouse model of neuropathic pain. Eur J Pharmacol 524: 80-83, 2005.
18. Mao J: Central glucocorticoid receptor: A new role in the cellular mechanisms of neuropathic pain. Rev Neurosci 16: 233-238, 2005.
19. Lu Y, Xiong X, Wang X, Zhang Z, Li J, Shi G, Yang J, Zhang H, Ning G and Li X: Yin yang 1 promotes hepatic gluconeogenesis through upregulation of glucocorticoid receptor. Diabetes 62: 1064-1073, 2013.
20. Bergad PL, Towle HC and Berry SA: Yin-yang 1 and glucocorticoid receptor participate in the Stat5-mediated growth hormone response of the serine protease inhibitor 2.1 gene. J Biol Chem 275: 8114-8120, 2000.
21. Wei N, Wang Y, Xu RX, Wang GQ, Xiong Y, Yu TY, Yang GS and Pang WJ: PU.1 antisense lncRNA against its mRNA translation promotes adipogenesis in porcine preadipocytes. Anim Genet 46: 133-140, 2015.
22. Wu J, Zhao Z, Zhu X, Renn CL, Dorsey SG and Faden AI: Cell cycle inhibition limits development and maintenance of neuropathic pain following spinal cord injury. Pain 157: 488-503, 2016.
23. Sato KL, Johanek LM, Sanada LS and Sluka KA: Spinal cord stimulation reduces mechanical hyperalgesia and glial cell activation in animals with neuropathic pain. Anesth Analg 118: 464-472, 2014.
24. Wang M, Liu C, Su Y, Zhang K, Zhang Y, Chen M, Ge M, Gu L, Lu T, Li N, *et al*: miRNA-34c inhibits myoblasts proliferation by targeting YY1. Cell Cycle 16: 1661-1672, 2017.
25. Ramkumar C, Cui H, Kong Y, Jones SN, Gerstein RM and Zhang H: Smurf2 suppresses B-cell proliferation and lymphomagenesis by mediating ubiquitination and degradation of YY1. Nat Commun 4: 2598, 2013.
26. Lu S, Wang MS, Chen PJ, Ren Q and Bai P: MiRNA-186 inhibits prostate cancer cell proliferation and tumor growth by targeting YY1 and CDK6. Exp Ther Med 13: 3309-3314, 2017.
27. Wang M, Zhi LI, Jinyu XU and Shanshan XU: The inhibition of cell proliferation and invasion through miR-34a regulating YY1 in human renal carcinoma cell. Lab Med 27: 635-640, 2012.

28. Riman S: Mechanisms regulating YY1 cleavage during apoptosis. Dissertations & Theses-Gradworks, 2011.
29. Yakovleva T, Kolesnikova L, Vukojević V, Gileva I, Tan-No K, Austen M, Lüscher B, Ekström TJ, Terenius L and Bakalkin G: YY1 binding to a subset of p53 DNA-target sites regulates p53-dependent transcription. Biochem Biophys Res Commun 318: 615-624, 2004.
30. Reséndizmartínez J, Asbunbojalil J, Huertayepez S and Vega M: Correlation of the expression of YY1 and fas cell surface death receptor with apoptosis of peripheral blood mononuclear cells and the development of multiple organ dysfunction in children with sepsis. Mol Med Rep 15: 2433-2442, 2017.
31. Trabucco SE, Gerstein RM and Zhang H: YY1 regulates the germinal center reaction by inhibiting apoptosis. J Immunol 197: 1699-1707, 2016.
32. Nicolas CS, Mascia A, Bortolotto ZA, Doherty A, Csaba Z, Fafouri A, Dournaud P, Gressens P, Collingridge GL and Peineau S: The role of JAK-STAT signaling within the CNS. JAKSTAT 2: e22925, 2013.
33. Planas AM, Gorina R and Chamorro A: Signalling pathways mediating inflammatory responses in brain ischaemia. Biochem Soc Trans 34: 1267-1270, 2006.
34. Cai QW, Li J, Li XQ, Wang JQ and Huang Y: Expression of STAT3, MMP-1 and TIMP-1 in gastric cancer and correlation with pathological features. Mol Med Rep 5: 1438-1442, 2012.
35. Matsukawa A, Kudo S, Maeda T, Numata K, Watanabe H, Takeda K, Akira S and Ito T: Stat3 in resident macrophages inflammatory response. J Immunol 175: 3354-3359, 2005.
36. Kiguchi N, Saika F, Kobayashi Y, Ko MC and Kishioka S: TC-2559, an α4β2 nicotinic acetylcholine receptor agonist, suppresses the expression of CCL3 and IL-1β through STAT3 inhibition in cultured murine macrophages. J Pharmacol Sci 128: 83-86, 2015.
37. Rummel C, Sachot C, Poole S and Luheshi GN: Circulating interleukin-6 induces fever through a STAT3-linked activation of COX-2 in the brain. Am J Physiol Regul Integ Comp Physiol 291: R1316-R1326, 2006.
38. Aid S, Langenbach R and Bosetti F: Neuroinflammatory response to lipopolysaccharide is exacerbated in mice genetically deficient in cyclooxygenase-2. J Neuroinflammation 5: 1-14, 2008.
39. Li L and Zhao G: Downregulation of microRNA-218 relieves neuropathic pain by regulating suppressor of cytokine signaling 3. International J Mol Med 37: 851-858, 2016.
40. Croker BA, Krebs DL, Zhang JG, Wormald S, Willson TA, Stanley EG, Robb L, Greenhalgh CJ, Förster I, Clausen BE, *et al*: SOCS3 negatively regulates IL-6 signaling in vivo. Nat Immunol 4: 540-545, 2003.
41. Xiang-Mei YU, Yang YZ, Liu JB, Chang-Zheng LI, Gong DG and Wang ZF: Effects of warming acupuncture therapy on expressions of IL-6 and SOCS3 in spinal cord in rats with neuropathic pain. Chin J Inform Tradit Chin Med, 2018.