

# Systemic Injection of Thalidomide Prevent and Attenuate Neuropathic Pain and Alleviate Neuroinflammatory Response in the Spinal Dorsal Horn

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**Background and objective:** Thalidomide (Tha) has been shown to exert immunomodulatory and anti-inflammatory properties. Whether Tha can alleviate spinal nerve ligation (SNL)-induced neuropathic pain (NP) is still unclear. This study aimed to investigate the therapeutic effect of Tha on the SNL-induced NP and further explore the potential analgesic mechanisms of Tha.

**Methods:** The effects of Tha on SNL-induced mechanical allodynia were assessed by pain behavioral testing. The expressions of the astrocyte marker glial fibrillary acidic protein (GFAP) and the microglia marker Iba1 in the spinal dorsal horn were evaluated by immunofluorescence histochemistry. Protein expressions of the tumor necrosis factor alpha (TNF- $\alpha$ ) in the spinal dorsal horn were tested by Western blot assay. Data were analyzed using one-way ANOVA or two-way ANOVA.

**Results:** By the pretreatment with a single intraperitoneal injection, the PWMT in SNL+Tha group was significantly increased from day 1 to day 2 after SNL ( $P < 0.05$  compared with SNL+Veh group). By the posttreatment with a single intraperitoneal injection, the PWMT in SNL+Tha group was also significantly increased from day 3 to day 4 after SNL ( $P < 0.05$  compared with SNL+Veh group). By the posttreatment with multiple intraperitoneal injection, both the PWMT and the PWTL in SNL+Tha group were similarly significantly increased from day 3 to day 14 after SNL ( $P < 0.05$  compared with SNL+Veh group). Furthermore, the GFAP and Iba1 expressions and TNF- $\alpha$  levels of the ipsilateral spinal dorsal horn in SNL+Tha group were significantly weaker from day 3 to day 14 after SNL than those in SNL+Veh group ( $P < 0.05$ ).

**Conclusion:** Tha can significantly alleviate NP induced by SNL. The analgesic mechanism may be related to inhibition of astrocyte and microglia activation as well as down-regulation of TNF- $\alpha$  levels in the spinal dorsal horn.

**Keywords:** Tha, neuropathic pain, Glia, TNF- $\alpha$ , spinal nerve ligation

## Introduction

Neuropathic pain (NP) is a severe public health problem that affects 6–8% of the world's adult population.<sup>1</sup> Effective treatment for NP is still lacking, in part due to poor understanding of the pathological mechanisms at the molecular level. Piles of evidence have revealed that neurons are not the only cell type involved in NP. Glial cells, including astrocytes and microglia, are emerging additional players in the initiation and maintenance of NP. These glial cells have close interactions with

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neurons and thus modulating pain transmission, particularly under pathological conditions. Therefore, inhibition of glial cell activation or blocking the release of cytokines from these cells constitute a potential therapeutic approach for NP.<sup>2,3</sup>

Thalidomide (Tha) was initially used as a sedative, tranquilizer and antiemetic for emesis gravidarum.<sup>4</sup> It was withdrawn from the market in 1965 because of the congenital defects that occurred when taken during gestation.<sup>5</sup> However, Tha has been shown to exert immunomodulatory and anti-inflammatory properties.<sup>6</sup> Multiple studies have indicated that Tha has significant promise in the treatment of various immunological disorders including microsporidial diarrhea,<sup>7</sup> bacterial meningitis,<sup>8</sup> chronic graft-vs-host disease,<sup>9</sup> Crohn's disease,<sup>10</sup> and septic shock.<sup>11</sup> And it is approved by the FDA for the treatment of multiple myeloma and leprosy as well. Although NP is also associated with an immunological disorder in the CNS,<sup>12</sup> whether Tha could alleviate NP remains unclear. Tha can significantly inhibit activation of both astrocytes and microglia in a mouse model of Alzheimer's disease<sup>13</sup> and suppress the production of tumor necrosis factor alpha (TNF- $\alpha$ ).<sup>14</sup> Currently, available evidence implies that Tha might provide a novel approach to the treatment of NP. In the present study, the spinal nerve ligation (SNL) rat model was used to assess the therapeutic effect of Tha on NP. The analgesic mechanism of Tha was explored which might involve both astrocytes and microglia and the cytokines released from the glial cells such as TNF- $\alpha$ .

## Materials and Methods

### Animal Preparation and Ethics Statement

Adult male Sprague–Dawley rats, which were purchased from the Fourth Military Medical University, weighing 200–220 g were housed in cages at room temperature of 22–26°C, with free access to food and tap water and were kept on a 12-hr, light–dark cycle. Before the behavioral experiment, animals were placed in the behavioral test environment for 30 mins for a total of 3 days. The animal experiments were conducted in accordance with protocols approved by the Animal Care and Use Committee at the Fourth Military Medical University (Xi'an, People's Republic of China; protocol No. FMMULL-20130136; approved on 6 March 2013; Chairperson: Dr Qian Wang). The ethical guidelines of this agreement were consistent with the National Institutes of Health and the International Association for the Study of Pain. The

experiment was carried out using the double-blind method, and efforts were made to minimize the number of animals and their suffering.

### Reagent Preparation

Reagents were Tha (H32026129, Changzhou Pharmaceutical Factory, Changzhou, China), DMSO (vehicle of Tha), monoclonal mouse anti-glial fiber acidic protein (GFAP) antibody (1:5000; MAB3402, Merck Millipore, Billerica, MA, USA), polyclonal goat anti-ionized calcium-binding adapter molecule 1 (Iba1) antibody (1:500; ab5076, Abcam, Cambridge, UK), polyclonal goat anti-tumor necrosis factor TNF- $\alpha$  antibody (1:100; sc-1351, Santa Cruz, CA, USA), alexa-594-labelled donkey anti-mouse IgG (1:500; A21203, Invitrogen, Carlsbad, CA, USA), alexa-594-labelled donkey anti-goat IgG (1:500; A11058, Invitrogen, Carlsbad, CA, USA), horseradish peroxidase (HRP)-labeled rabbit anti-goat IgG (ZB-2306, Zhongshan Co, Beijing, China).

### Experimental Design

Firstly, to verify the effect of Tha pretreatment induced by SNL, 12 rats were divided into 2 groups: SNL+Veh group and SNL+Tha group (n = 6). Drugs were injected 20 mins before SNL with a single intraperitoneal injection. Secondly, to verify the effect of Tha posttreatment induced by SNL, 12 rats were divided into 2 groups: SNL+Veh group and SNL+Tha group (n = 6). Drugs were injected 3 days after SNL with a single intraperitoneal injection. Thirdly, to verify the effect of Tha posttreatment induced by SNL with multiple intraperitoneal injections, 12 rats were divided into 2 groups: SNL+Veh group and SNL+Tha group (n = 6). Drugs were injected at the 3rd, the 5th, the 7th and the 14th day after SNL, respectively. Pain behavior was assessed by the paw withdrawal mechanical threshold (PWMT) and paw withdrawal thermal latency (PWTl) tests 30 mins after injection once daily on SNL 0, 1, 2, 3, 4, 5, 6, 7 and 14 days. At last, to further verify the role of astrocyte activation, microglial activation and the expression of TNF- $\alpha$  in the spinal dorsal horn of Tha posttreatment with multiple intraperitoneal injections, 96 rats were divided into 4 groups: Sham+Veh group (n = 12), Sham+Tha group (n=12), SNL+Veh group (n = 36) and SNL+Tha group (n = 36). In each group, half of the rats were used for immunofluorescence histochemistry of the astrocyte marker glial fibrillary acidic protein (GFAP) and the microglia marker Iba1 and the other rats were used for Western blot assay of the tumor necrosis factor alpha (TNF- $\alpha$ ) at the 3rd, the 7th and the 14th day after SNL,

respectively ( $n = 6$ ). The SNL+Tha group and Sham+Tha group received 20 mg/kg Tha, and the Sham+Veh group and SNL+Veh group received an equal volume of vehicle.

## SNL Model

All the rats were given an intraperitoneal injection of 7% chloral hydrate 2 mL/kg. The skin of the backside of the iliac crest was disinfected with 75% alcohol, and then we made a longitudinal skin incision of about 3 cm located 0.5 cm left of the posterior midline. The transverse process of left L5 was removed to make clear the L4 and L5 spinal nerves. Then, the left L5 spinal nerve was separated and carefully ligated with 6-0 suture. Then, each opened layer was closed with a suture, including the muscle and skin. And except the spinal nerves were not ligated, the sham group rats have the same surgical procedure to that of SNL group rats. After the rats were completely awake, they were replaced in their cages for conventional feeding.

## Von Frey Test

Mechanical sensitivity was assessed by means of the electronic von Frey test. The test rats were placed on an elevated metal mesh floor and put in Plexiglas boxes ( $50 \times 30 \times 30$  cm). The electronic von Frey filaments were pressed on the plantar surface for about 5 s, which was repeated 3 times at a 3-m interval on each hind paw. The mean value of the three results was considered as the paw withdrawal mechanical threshold (PWMT). PWMT tests were firstly performed 1 hr before SNL to get a baseline and repeated every day in a week after SNL.

## Hargreaves Test

Nociceptive responsiveness to heat was evaluated using a modified version of the Hargreaves test. Briefly, the rats were placed in clear plastic chambers ( $30 \times 30 \times 30$  cm) having thick, transparent glass floors that were suspended 15 cm from the top of the bench. The rats were allowed to habituate to the apparatus for 30 mins before testing. The set voltage of the RTY-3 10-V and 100-W halogen projector lamp was applied as a source of heat pain. By adjusting the distance between the glass plate and the light source, thermal radiation having a diameter of 5 mm was applied to the plantar surface of each rat's paw. Observation and recording of the time from the beginning of thermal radiation to the reduction in foot reflex time was recorded in seconds, with 20 s for each test interval. The measurements were repeated five times; the average number of Hargreaves test values was recorded as paw withdrawal

thermal latency (PWTL). A cut-off time of 40 s was adopted to avoid possible tissue damage, and the average value was recorded as 30 s.

## Immunofluorescence Histochemistry

Rats were anaesthetized by 7% hydrate 3 mL/kg intraperitoneal injection, from the aorta perfused with 100 mL 0.9% normal saline to wash the blood quickly after opening the chest, followed by 500 mL 4% paraformaldehyde-phosphate buffer (pH 7.4), and continuously perfused for 2 hrs. After decapitation, the whole spinal cord was removed and immersed in the same fixative for 4 hrs at room temperature. The tissues were then stored overnight in 30% sucrose solution in 0.1 mol/L phosphate buffer at 4°C for cryoprotection. The spinal cord was sliced into 30- $\mu$ m-thick sections with a cryotome (CN1800, Leica, Wetzlar, Germany) at -20°C. The sections were collected in sterile 0.01 mol/L phosphate buffer (pH 7.4) at 4°C and processed for GFAP and Iba1 immunofluorescence histochemistry. The free-floating sections were rinsed in 0.01 mol/L phosphate buffer saline (PBS) (pH 7.4) three times (10 mins each) and then sliced into 0.3% TritonX-100 and 2% sheep serum 0.01 PBS mol/L at room temperature for 1 hr and then incubated overnight at 4°C with either monoclonal mouse anti-GFAP antibody (diluted 1:5000) or polyclonal goat anti-ionized calcium-binding adapter molecule 1 (diluted 1:500). The sections were then washed with PBS ( $3 \times 10$  mins) and incubated with either biotinylated alexa-594-labelled donkey anti-mouse IgG or alexa-594-labelled donkey anti-goat IgG secondary antibody (diluted 1:500) for 4 hrs. Avoiding light, we washed the sections with PBS ( $3 \times 10$  mins). After staining, all of the sections were mounted onto glass slides and cover-slipped with 50% (v/v) glycerol and 2.5% (w/v) were mounted onto glass slides and cover-slipped triethylenediamine (anti-fading agent) in 0.05 M PBS. After being dried with fluorescent mounting medium mounting, the sections were mounted onto gelatin-coated glass slides.

## Western Blot Analysis

Western blot analysis was used to detect the expression of TNF- $\alpha$  in the samples as described previously. Briefly, the rats were deeply anesthetized by intraperitoneal injection of 7% chloral hydrate. Then, they were decapitated and the L5 segmental spinal cord was removed quickly and placed on ice. Total protein from each group was acquired using an extraction kit (KeyGen, Nanjing, China) on ice. The total protein concentration of the samples was analyzed with a BCA kit (Sigma-Aldrich, St Louis, Mo, USA). The

following primary antibodies were used: polyclonal goat anti-tumor necrosis factor TNF- $\alpha$  antibody (1:100; Santa Cruz) and secondary horseradish peroxidase (HRP)-labeled rabbit anti-goat IgG (ZB-2306, Zhongshan Co, Beijing, China). The signals were detected using an ECL kit (Pierce) according to the manufacturer's instructions. The changes in relative protein expression were represented as the ratio of the integrated optical density of the protein bands to that of  $\beta$ -actin. Quantitative analysis of the protein bands was performed using an Image-Quant 5.0 GE Healthcare Densitometer (GE Healthcare, Sunnyvale, CA, USA). The experiments were performed independently in triplicate.

## Immunohistochemical Image Analysis

Five sections from the spinal cord of each rat were selected randomly, using a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan) to observe the spinal dorsal horn and analyse the images, and the sections were observed with the appropriate laser beams and filter settings for red-emitting Alexa 594 (excitation, 543 nm; emission, 590 to 615 nm). The digital images were captured using FV10-ASW-1.6 software (Olympus, Tokyo, Japan), modified (15% to 20% contrast enhancement) in Photoshop CS4 (Adobe Systems, San Jose, CA, USA) and then saved as TIFF files.

All stained sections were examined and analyzed by an investigator who was blinded to the treatment.

## Statistical Analysis

The statistical analysis was performed with SPSS 24.0 for Windows (SPSS, Inc., IBM) and Graphpad Prism 7 (GraphPad Software, La Jolla, CA, USA). Two-way repeated analyses of variance (ANOVAs) were used to compare two groups in different time points in von Frey test and Hargreaves test. Data from the immunofluorescence histochemistry and Western blot were analyzed using a one-way ANOVA followed by the least significant difference (LSD) for post hoc analysis. All values were presented as the means  $\pm$  SEM. Values of  $P < 0.05$  were considered statistically significant.

## Results

### Effect of Tha Pretreatment on the PWMT Induced by SNL with a Single Intraperitoneal Injection in Rats

SNL (L5) decreased the PWMT significantly from  $37.4 \pm 4.6$  g presurgery to  $12.8 \pm 1.3$  g after the surgery. The PWMT was

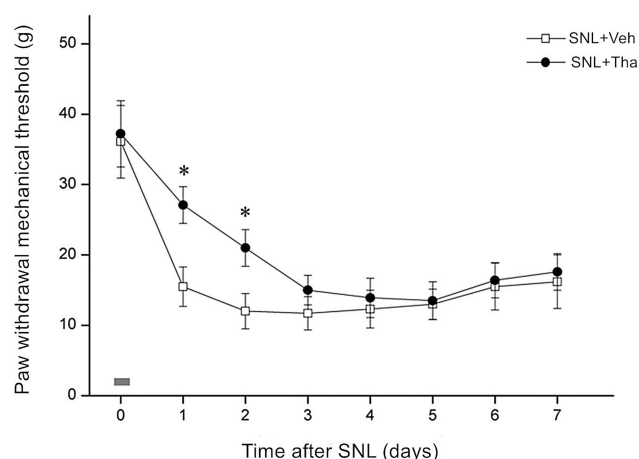
then gradually increased to  $17.1 \pm 1.5$  g by day 7. Rats in SNL+Tha group were treated with an intraperitoneal injection of 20 mg/kg Tha 20 mins before SNL. The PWMT decreased significantly during the first week, whereas the PWMT was higher in SNL+Tha group than in SNL+Veh group at the 1st day and 2nd day after surgery ( $P < 0.05$ ), and there was no statistical difference between SNL+Tha group and SNL+Veh group from the 3rd to the 7th day ( $P > 0.05$ ), indicating that the Tha pretreatment had an effect on PWMT change induced by the SNL lasting about 3 days (Figure 1).

### Effect of Tha Posttreatment on the PWMT Induced by SNL with a Single Intraperitoneal Injection in Rats

The PWMT decreased significantly in both SNL+Tha group and SNL+Veh group after SNL, and there was no difference between the two groups ( $P > 0.05$ ). Rats in SNL+Tha group were treated with an intraperitoneal injection of 20 mg/kg Tha 3 days after SNL. The PWMT in the SNL+Tha group was increased rapidly from  $13.4 \pm 1.6$  g to  $27.2 \pm 1.5$  g 30 mins after injection, which was higher than that of SNL+Veh group ( $14.7 \pm 1.1$ g,  $P > 0.05$ ) and lasted for 2 days (Figure 2).

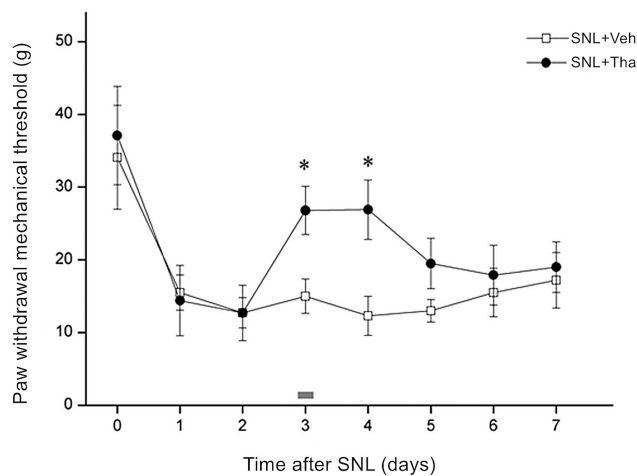
### Effect of Tha Posttreatment on the PWMT Induced by SNL with Multiple Intraperitoneal Injections in Rats

According to the above results, 20 mg/kg Tha was intraperitoneally injected at the 3rd, the 5th, the 7th and the 14th day



**Figure 1** Effect of Tha pretreatment on the PWMT induced by SNL with a single intraperitoneal injection in rats ( $n = 6$ ,  $\bar{x} \pm s$ ). Gray box marks the time point for the drug administration. \* $P < 0.05$ , compared with SNL+Veh group. Two-way repeated analyses of variance (ANOVAs) were used for statistical analysis.

**Abbreviations:** PWMT, paw withdrawal mechanical threshold; SNL, spinal nerve ligation; Tha, Thalidomide; Veh, vehicle.



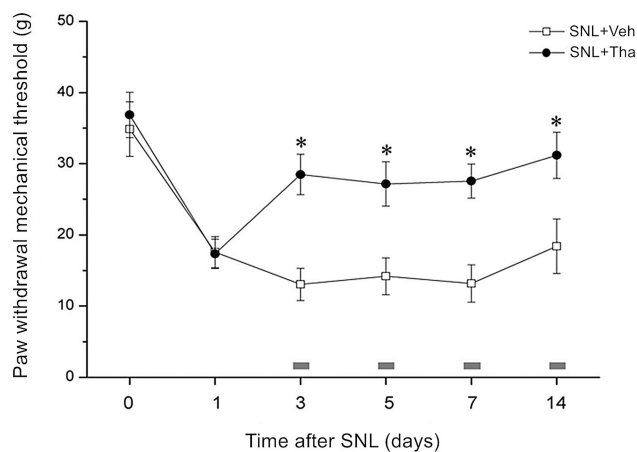
**Figure 2** Effect of Tha posttreatment on the PWMT induced by SNL in rats ( $n = 6$ ,  $\bar{x} \pm s$ ) with a single intraperitoneal injection. Gray box marks the time point of drug administration.  $*P < 0.05$ , compared with SNL+Veh group. Two-way repeated analyses of variance (ANOVAs) were used for statistical analysis.

**Abbreviations:** PWMT, paw withdrawal mechanical threshold; SNL, spinal nerve ligation; Tha, Thalidomide; Veh, vehicle.

after SNL in SNL+Tha group, respectively. The PWMTs were higher from days 3 to 14 in SNL+Tha group than in SNL+Veh group at every testing time point ( $P < 0.05$ , Figure 3).

### Effect of Tha Posttreatment on the PWTL Induced by SNL with Multiple Intraperitoneal Injections in Rats

The trends of the thermal PWTLs were similar to those of the PWMTs. The PWTLs were decreased on the first day after



**Figure 3** Effect of Tha posttreatment on the PWMT induced by SNL in rats ( $n = 6$ ,  $\bar{x} \pm s$ ) with multiple intraperitoneal injections. Gray box marks the time point for drug administration.  $*P < 0.05$ , compared with SNL+Veh group. Two-way repeated analyses of variance (ANOVAs) were used for statistical analysis.

**Abbreviations:** PWMT, paw withdrawal mechanical threshold; SNL, spinal nerve ligation; Tha, Thalidomide; Veh, vehicle.

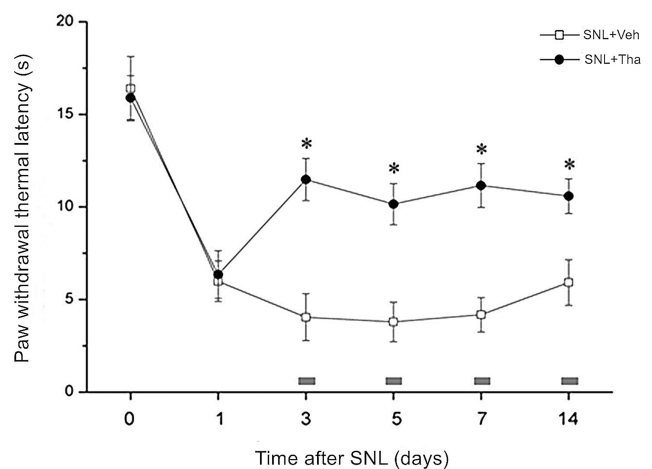
SNL in both SNL+Tha group and SNL+Veh group, and there was no difference between groups. An intraperitoneal injection of 20 mg/kg Tha was given at the 3rd, the 5th, the 7th and the 14th day after SNL in SNL+Tha group, respectively. The PWTLs were tested 30 mins after injection and were significantly higher in SNL+Tha group than in SNL+Veh group at every testing time point ( $P < 0.05$ , Figure 4).

### Effect of Tha Posttreatment with Multiple Intraperitoneal Injections on SNL-Induced Astrocytes Activation in the Spinal Dorsal Horn in Rats

GFAP expression in the ipsilateral spinal dorsal horn was higher in SNL+Veh group than in the sham group ( $P < 0.05$ ). The trend peaked on the 3rd day, and the increase lasted for 14 days after SNL. The SNL+Tha group received a 20 mg/kg injection of Tha at the 3rd, the 5th, the 7th and the 14th day after SNL, respectively. Immunofluorescence staining indicated that the expression of GFAP in the ipsilateral spinal dorsal horn was lower in SNL+Tha group compared with SNL+Veh group ( $P < 0.05$ , Figure 5).

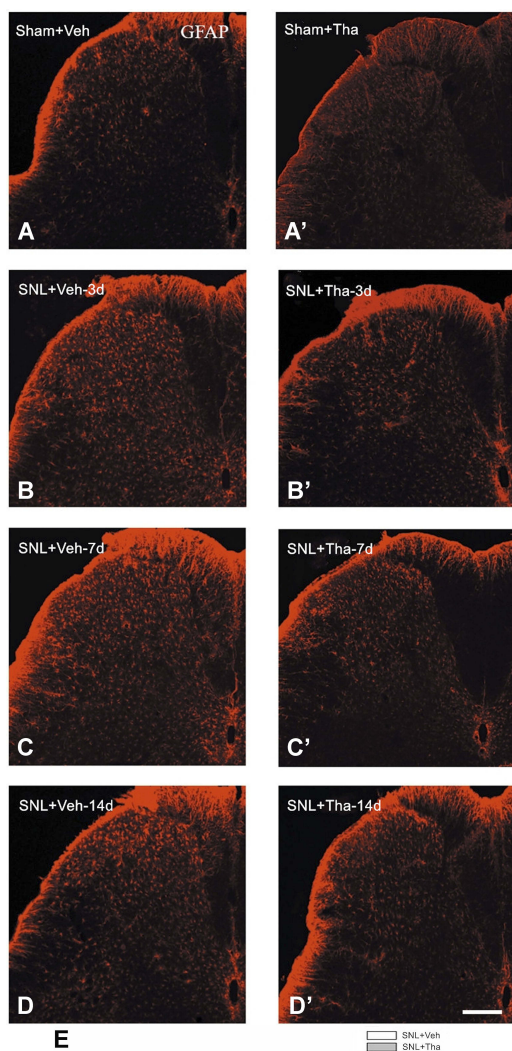
### Effects of Tha Posttreatment with Multiple Intraperitoneal Injections on SNL-Induced Microglial Activation in the Spinal Dorsal Horn in Rats

The Iba1 expression in the ipsilateral spinal dorsal horn was higher in SNL+Veh group than in the sham group ( $P < 0.05$ ),



**Figure 4** Effect of Tha posttreatment on the PWTL induced by SNL in rats ( $n = 6$ ,  $\bar{x} \pm s$ ) with multiple intraperitoneal injections. Gray box marks the time point for drug administration.  $*P < 0.05$ , compared with SNL+Veh group. Two-way repeated analyses of variance (ANOVAs) were used for statistical analysis.

**Abbreviations:** PWTL, paw withdrawal thermal latency; SNL, spinal nerve ligation; Tha, Thalidomide; Veh, vehicle.



**Figure 5** The picture shows the expression of GFAP, a marker of astrocytes, was down-regulated by Tha posttreatment (multiple intraperitoneal injections) on the left side of spinal dorsal horn of SNL rats. (n = 6,  $\bar{x} \pm s$ ). (A) The expression of GFAP on the left side of spinal dorsal horn of the Sham+Veh group rats. (B–D) The expression of GFAP on the left side of spinal dorsal horn of SNL+Veh group rats on the 3rd day, the 7th day and the 14th day, respectively, after SNL. (A') The expression of GFAP on the left side of spinal dorsal horn of the Sham+Tha group rats. (B'–D') The expression of GFAP in the left side of spinal dorsal horn of SNL+Tha group rats on the 3rd day, the 7th day and the 14th day, respectively, after SNL. Scale bar = 100  $\mu$ m. (E) <sup>#</sup>*p* < 0.05, compared with the sham group; <sup>\*</sup>*p* < 0.05, compared with the same time point of SNL+Veh group. One-way ANOVA followed by the least significant difference (LSD) for post hoc analysis was used for statistical analysis.

**Abbreviations:** GFAP, glial fibrillary acidic protein; SNL, spinal nerve ligation; Tha, Thalidomide; Veh, vehicle; SNL-3d, 3rd day after SNL; SNL-7d, 7th day after SNL; SNL-14d, 14th day after SNL.

which was at a high level on the 7th and 14th day after SNL. The SNL+Tha group was treated with a 20 mg/kg injection at

the 3rd, the 5th, the 7th and the 14th day after SNL, respectively. Immunofluorescence staining indicated that the expression of Iba1 on the ipsilateral spinal dorsal horn was lower in SNL+Tha group than in SNL+Veh group (*P* < 0.05, Figure 6).

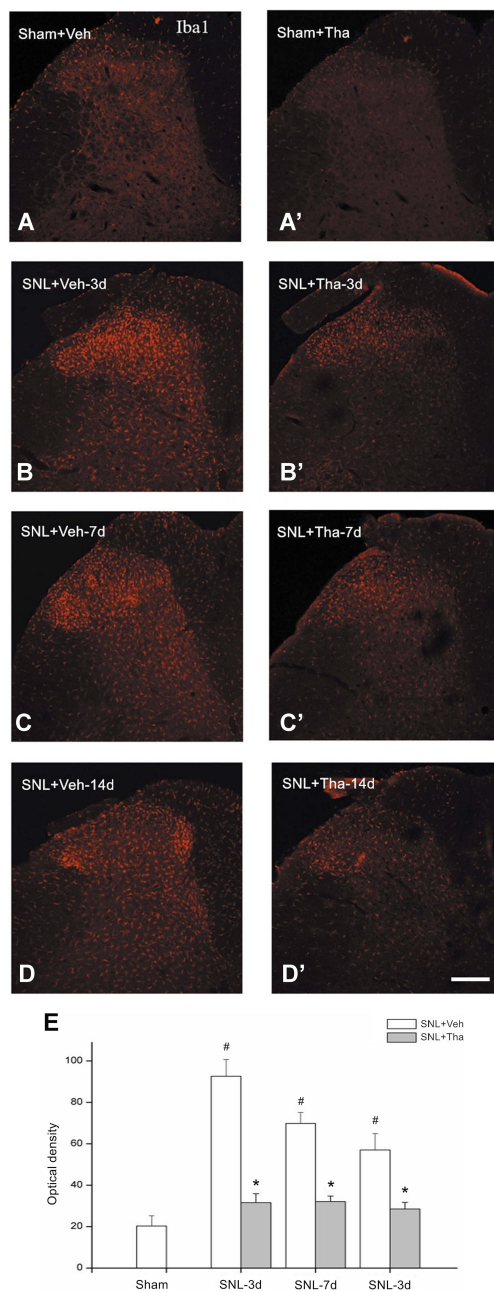
### Effect of Tha Posttreatment with Multiple Injections on the Expression of TNF- $\alpha$ in the Dorsal Horn of the Spinal Cord of Rats After SNL

TNF- $\alpha$  expression in the ipsilateral spinal dorsal horn was measured by Western blot analysis after injection of 20 mg/kg Tha at the 3rd, the 5th, the 7th and the 14th day after SNL, respectively. TNF- $\alpha$  expression in the SNL+Veh group was increased from  $25.7 \pm 3.8$  to  $62.7 \pm 4.5$  on the 3rd day, peaked on the 7th day ( $73.4 \pm 4.1$ ) and remained at a high level on the 14th day ( $60.1 \pm 4.7$ ). TNF- $\alpha$  expression was higher in SNL+Veh group than in the sham group at each time point (*P* < 0.05). TNF- $\alpha$  expression in SNL+Tha group at the 3rd, 7th and 14th day after SNL was  $38.2 \pm 3.2$ ,  $26.8 \pm 3.3$  and  $29.8 \pm 3.3$ , respectively, which was lower than in SNL+Veh group at the same time point (*P* < 0.05, Figure 7).

### Discussion

NP is the most common chronic pain found clinically. Research on targeted cells focuses mainly on the neurons as they are the only cells generating and propagating action potentials in the CNS.<sup>15</sup> However, recent studies have shown that the nociceptive stimulus can cause excessive activation of the glial cells in the spinal cord in NP models. Moreover, the nociceptive stimulus can not only increase the number of activated glial cells, but also change the cell morphology. Furthermore, it has been determined that excessive activation of microglia occurs earlier than that of astrocytes. Excessive activation of microglia and astrocytes is closely related to the occurrence and maintenance of NP.<sup>16,17</sup> Studies have confirmed that NP can be relieved by inhibiting excessive activation of glial cells in the central and peripheral nervous systems.<sup>18</sup>

The pharmacotherapy of neuropathic pain is challenging and many analgesics have adverse effects, which limited their usage. First-line drugs for NP include antidepressants and anticonvulsants. The antidepressants include tricyclic antidepressants (TCAs) and serotonin-noradrenaline reuptake inhibitors. The TCAs have many



**Figure 6** The expression of Iba1, a marker of microglia, was down-regulated by Tha posttreatment (multiple intraperitoneal injections in left side of spinal dorsal horn of SNL rats) ( $n = 6, \bar{x} \pm s$ ). (A) The expression of Iba1 on the left side of spinal dorsal horn of the Sham+Veh group rats. (B–D) The expression of Iba1 on the left side of spinal dorsal horn of SNL+Veh rats on the 3rd day, the 7th day and the 14th day, respectively, after SNL. (A') The expression of Iba1 on the left side of spinal dorsal horn of the Sham+Tha group rats. (B'–D') The expression of Iba1 in the left side of spinal dorsal horn of SNL+Tha rats group on the 3rd day, the 7th day and the 14th day, respectively, after SNL. Scale bar = 100  $\mu\text{m}$ . (E)  $^{\#}P < 0.05$ , compared with the sham group;  $^*P < 0.05$ , compared with the same time point of SNL+Veh group. One-way ANOVA followed by the least significant difference (LSD) for post hoc analysis was used for statistical analysis.

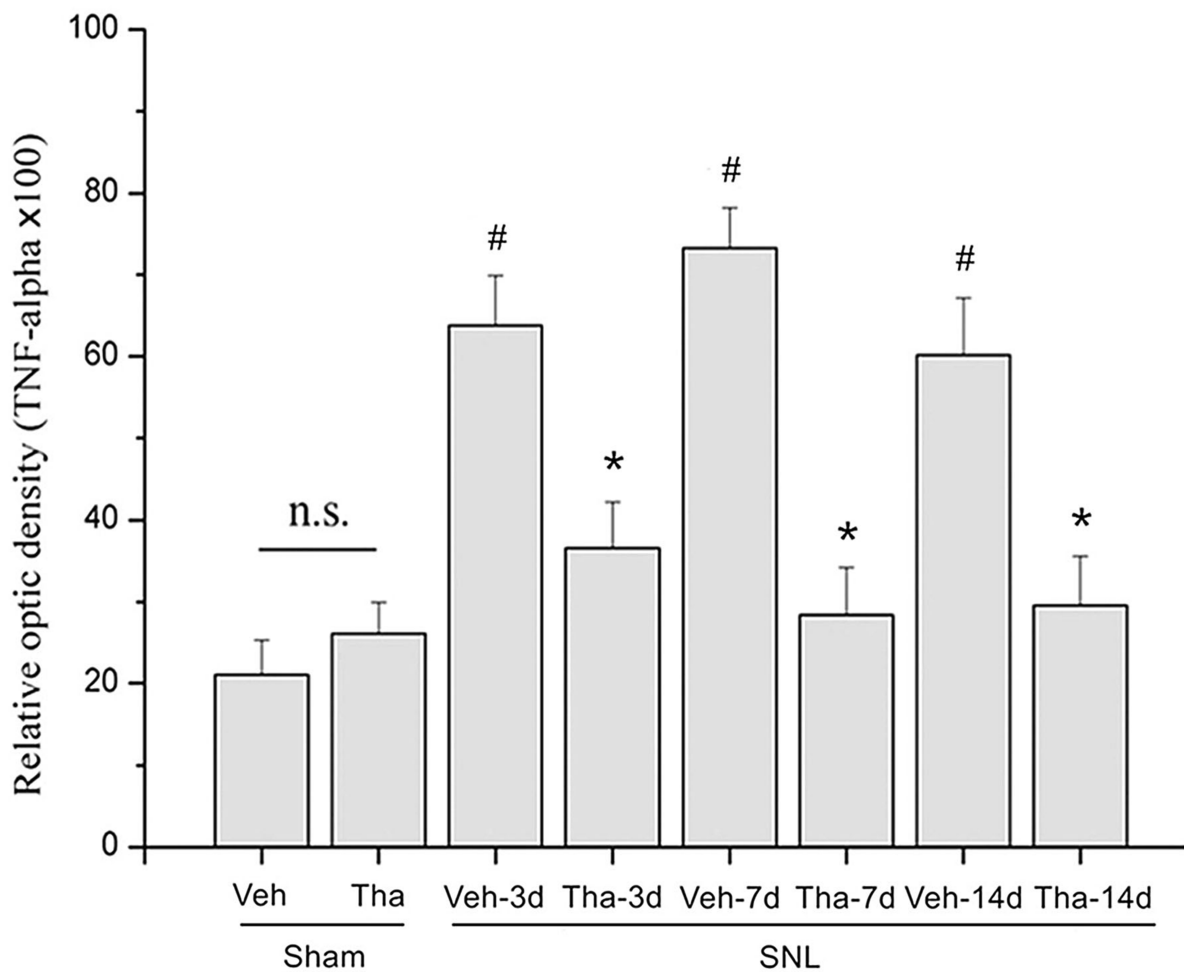
**Abbreviations:** Iba1, ionized calcium-binding adapter molecule 1; SNL, spinal nerve ligation; Tha, Thalidomide; Veh, vehicle; SNL-3d, 3rd day after SNL; SNL-7d, 7th day after SNL; SNL-14d, 14th day after SNL.

adverse drug reactions such as dry mouth, orthostatic hypotension, constipation and urinary retention. What is

more, the anticholinergic effects of TCAs increase the risk of cardiotoxicity. Duloxetine is one of the serotonin-norepinephrine reuptake inhibitors, the most common adverse effect of which is nausea. The anticonvulsants, such as pregabalin and gabapentin exert their analgesic effects by acting at calcium channels. However, they may lead to dizziness as well as somnolence. Second- and third-line drugs for NP include topical lidocaine and opioids. As lidocaine is usually applied topically, the most common adverse effects of lidocaine are mild local reactions. Clinical experiments have shown that opioids have serious side effects. In addition, long-term application of opioids may lead to addiction.<sup>19–22</sup> Effective treatment is still lacking for many NP patients. Therefore, new analgesics are needed for the successful cure of NP.

Tha, produced by Grunenthal GmbH in the 1950s, is a synthetic derivative of glutamic acid with sedative-hypnotic and analgesic properties. The effect of Tha on pregnancy nausea and vomiting was so effective that its use quickly spread all over the world. However, within a few years, Tha caused devastating teratogenic effects and was explicitly banned. Since the removal of Tha from the market, its immunomodulatory, anti-inflammatory and anti-angiogenic effects were discovered successively.<sup>23,24</sup> Research on Tha has achieved encouraging results and is being given great importance. In recent studies, the effect of Tha on relieving peripheral nerve injury and cancer pain has been confirmed. However, the mechanism has not been fully elucidated.<sup>25,26</sup>

Our results showed that the PWMT of affected sides remarkably decreased after L5 ligation and peaked at 3 days postoperation and lasted 14 days. In the present study, Tha was used as a drug intervention. A single injection of Tha exerted an analgesic effect that lasts for 2 days (Figures 1 and 2). While a series injection of Tha might prolong its analgesic effect (Figures 3 and 4). The mechanism underlying the Tha analgesia effect in the present study might be owned to its immunomodulatory effect. Our results showed that the expression of GFAP, which can specifically label astrocytes, was significantly increased at the spinal dorsal horn of the injured side (Figure 5). The study also demonstrated that the expression of Iba1, which is the microglial marker, was increased at the spinal dorsal horn of the injured side (Figure 6). The expression of GFAP and Iba1 was in accordance with the pain response on the SNL side in the spinal dorsal horn. The level of TNF- $\alpha$  was increased at 3 days postoperatively and the high level lasted up to 14 days (Figure 7). These results revealed the activation of astrocytes and



**Figure 7** Effects of Tha posttreatment with multiple intraperitoneal injections on the expression of TNF- $\alpha$  in the dorsal horn of the spinal cord of rats after SNL (n = 6,  $\bar{x} \pm s$ ). #P < 0.05, compared with the sham group; \*P < 0.05, compared with the same time point of SNL+Veh group. One-way ANOVA followed by least significant difference (LSD) for post hoc analysis was used for statistical analysis.

**Abbreviations:** TNF- $\alpha$ , tumor necrosis factor alpha; SNL, spinal nerve ligation; Tha, Thalidomide; Veh, vehicle; SNL+Veh-3d, 3 days after SNL in rats by intraperitoneal injection of the same amount of vehicle; SNL+Tha-3d, 3 days after SNL in rats by intraperitoneal injection of Tha; SNL+Veh-7d, 7 days after SNL in rats by intraperitoneal injection of the same amount of vehicle; SNL+Tha-7d, 7 days after SNL in rats by intraperitoneal injection of Tha; SNL+Veh-14d, 14 days after SNL in rats by intraperitoneal injection of the same amount of vehicle; SNL+Tha-14d, 14 days after SNL in rats by intraperitoneal injection of Tha.

microglia, and the increasing release of TNF- $\alpha$  was almost simultaneous with the decrease of ipsilateral mechanical allodynia and shortening of thermal latency. Various NP models have confirmed that activation of astrocytes and microglia has a close relationship with pain behavior and can induce the release of pain-associated active substances, including excitatory amino acids, neuropeptides,

nitrous oxide and inflammatory factors. The active substances can also feedback onto the astrocytes and microglia, forming a vicious circle, which may be relieved or blocked by the inhibitors of astrocytes and microglia.<sup>27,28</sup> It is speculated that the NP may be relieved by controlling excessive activation of the glial cells and the effective release of inflammatory cytokines in the present study.



Although Tha can act as an analgesic for NP, it has severe side effects. The most common side effect of Tha is peripheral neuropathy, which may decrease the quality of patients' life. The underlying mechanisms of Tha-induced peripheral neuropathy (TIPH) remain not fully understood. But it has been proposed that the immunomodulatory effect may contribute to TIPN. Tha can reduce neuronal cell survival by the downregulation of TNF- $\alpha$  and the inhibition of NF- $\kappa$ B, resulting in the dysregulation of neurotrophins and their receptors and the subsequent acceleration of neuronal cell death. Moreover, the anti-angiogenic effect induced by Tha causes secondary ischemia and hypoxia of nerve fibers and subsequently irreversible damage to sensory neurons. The mechanism of Tha-induced teratogenesis is that the dihydroxy metabolite of Tha was capable of causing extensive redox-activated DNA cleavage. Whether or not the mechanism is also responsible for TIPH is yet to be confirmed by further preclinical and clinical trials.<sup>29</sup> However, piles of evidence have confirmed a dose- and time-dependent manner of TIPN development.<sup>30</sup> Thus, Tha may be used in the treatment of neuropathic pain carefully with a low dose and short duration. The effectiveness of the strategy needs further support from clinical trials.

In conclusion, the present study showed that Tha relieved the mechanical allodynia and thermal hyperalgesia of NP induced by SNL, suggesting that Tha is an effective analgesic for NP. Furthermore, the excessive activation of astrocytes and microglia and the excessive release of TNF- $\alpha$  have a close relationship with the occurrence, level and duration of NP induced by SNL. The analgesic mechanism of Tha could be mediated by inhibiting the excessive activation of glia cells and reducing the undue release of TNF- $\alpha$ .

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## Disclosure

The authors report no conflicts of interest in this work.

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