

Experimental Trial

Triptolide Prevents and Attenuates Neuropathic Pain via Inhibiting Central Immune Response

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Background: Current treatments for neuropathic pain are far from satisfactory. Considering the essential contribution of central immune factors to the pathogenesis of neuropathic pain, targeting inflammatory response is well accepted as an effective strategy for treating neuropathic pain. Triptolide has a long history in traditional Chinese medicine for treating inflammatory diseases and has been proven to inhibit cytokines released from glial cells.

Objective: In the present study, we tested whether systemic treatment with triptolide could prevent or attenuate nocifensive behaviors associated with neuropathic pain. We further tried to explore the underlying mechanism of the potential anti-allodynia effect of triptolide.

Study Design: A randomized, double blind, controlled animal trial.

Methods: Triptolide was administered systemically in a rat model of neuropathic pain induced by spinal nerve ligation (SNL) in the single bolus and repeated treatment manners. In the single bolus treatment experiment, triptolide (30 µg/kg, 100 µg/kg, 300 µg/kg) or vehicle was given to SNL and sham-operated rats once on day 1 or on day 10 after surgery (n = 6 each). In the repeated treatment study, prophylactic treatment with triptolide (30 µg/kg, 100 µg/kg, 300 µg/kg) was given to rats during the period of day -3 (3 days prior to SNL) to day 7 (7 days post-SNL) inclusively (n = 6 each). Another set of SNL and sham rats on postoperative day 10 received treatment with triptolide (30 µg/kg, 100 µg/kg, 300 µg/kg) or vehicle during the period of days 11–20 inclusively (n = 6 each), to assess potential reversal of established pain behavior. Mechanical allodynia of the rats was tested with von Frey filaments. Astrocytic and microglial activation in the spinal dorsal horn was evaluated with immunofluorescent histochemistry. Phosphorylation of mitogen-activated protein kinases (MAPKs), and expression of inflammatory cytokines (interleukin-6, interleukin-1beta, monocyte chemoattractant protein-1, and tumor necrosis factor-alpha) were examined with Western blot analysis and real-time reverse transcription polymerase chain reaction study.

Results: A single bolus treatment with triptolide could neither prevent the induction nor reverse the maintenance of SNL-induced mechanical allodynia. However, repeated administration of triptolide dose-dependently inhibited neuropathic pain behavior in both preventative and interventional paradigms. Triptolide hampered SNL-induced activation of glial cells (astrocytes and microglia) in the spinal dorsal horn without influencing neurons. In addition, SNL-induced phosphorylation of MAPKs could be inhibited by triptolide. Furthermore, up-regulated expression of inflammatory cytokines in neuropathic pain states could be remarkably blocked by triptolide.

Limitations: The direct target site (such as a specific receptor) of triptolide is still to be determined. In addition, triptolide could not completely block the SNL-induced mechanical allodynia.

Conclusions: Our data suggest that triptolide may be a potential novel treatment for neuropathic pain through modulating immune response in the spinal dorsal horn.

Key words: Triptolide, neuropathic pain, spinal dorsal horn, astrocyte, microglia, MAPK.

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Lesions in somatosensory pathways in the peripheral or central nervous system (CNS) cause neuropathic pain (1). Current treatments for neuropathic pain are far from being satisfactory (2). Over the past 2 decades, a surge of attention has developed about the involvement of immune response in neuropathic pain (3-7). Following treatments with toxins targeting glial cells, or treatments with antibodies/inhibitors against cytokines and chemokines, could significantly prevent and/or attenuate neuropathic pain behavior (8,9). However, the actions of these drugs are usually not confined to glial cells and may probably have toxic effects on neurons. In addition, some of these drugs present a high risk of either acute or cumulative toxicity, which could hamper their persistent usage. More importantly, the inflammatory response is mediated by cell-to-cell communication and a network of varieties of cytokines and chemokines (10,11). Targeting only one molecular pathway may not be sufficient for pain relief.

The Chinese herb *Tripterygium wilfordii* Hook. f. (TWHF) is a vinelike member of the Celastraceae plant family. As one of the major active ingredients of TWHF, triptolide has been used in traditional Chinese medicine for treating inflammatory diseases (12). Recent studies suggested that triptolide could inhibit tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, and nitric oxide production in microglia (13). Triptolide was also shown to inhibit the proliferation of reactive astrocytes and block the hypertrophy of astrocytic processes after spinal cord injury (14). Interestingly, administration of triptolide effectively protected neurons from inflammation-mediated damage through inhibiting microglial activation (15). These data indicated that triptolide could significantly repress the immune response of the CNS. However, it was still unknown whether triptolide had beneficial effects in treating neuropathic pain.

In the present study, we tested whether systemic treatment with triptolide could prevent or attenuate nocifensive behaviors associated with neuropathic pain. We further tried to explore the underlying mechanism of the potential anti-allodynia effect of triptolide.

METHODS

Animals and Surgery

Male Sprague-Dawley rats (180–220 g) were used in the present study. The spinal nerve ligation (SNL) model was built according to previous protocols (6,7,16). After anesthetized with pentobarbital (45 mg/kg, intraperi-

toneal), the left lumbar (L) 6 transverse process of the rat was removed. The L5 spinal nerve was then carefully isolated and tightly ligated with 6-0 silk thread. The surgical procedures for the sham group were identical to that of the SNL group, except that the spinal nerves were not ligated. All experimental procedures received prior approval as well as ethical guidelines to investigate experimental pain in conscious animals (17). All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

Administration of Drugs

Triptolide (Sigma, St. Louis, MO) was dissolved in 5% dimethylsulfoxide (DMSO [Sigma, St. Louis, MO]) at a concentration of one $\mu\text{g}/\mu\text{l}$. In the single bolus treatment experiment, triptolide (30, 100, 300 $\mu\text{g}/\text{kg}$) or vehicle (5% DMSO) was given to SNL and sham-operated rats once on day 1 or on day 10 after surgery ($n = 6$ rats per treatment group). The repetitive treatment experiment was further divided into prophylactic and reversal treatment. Prophylactic treatment was given to SNL and sham-operated rats in the form of triptolide (30 $\mu\text{g}/\text{kg}$, 100 $\mu\text{g}/\text{kg}$, 300 $\mu\text{g}/\text{kg}$) or vehicle during the period of day -3 (3 days prior to surgery) to day 7 (7 days postsurgery) inclusively ($n = 6$ rats per treatment group). Another set of SNL and sham rats on post-operative day 10 received treatment with triptolide (30 $\mu\text{g}/\text{kg}$, 100 $\mu\text{g}/\text{kg}$, 300 $\mu\text{g}/\text{kg}$) or vehicle during the period of days 11–20 inclusively ($n = 6$ rats per treatment group) to assess the potential reversal of established pain behavior.

Pain Behavior Test

The protocols for pain behavior tests were similar to previous reports (6,18). The rats were habituated to the testing environment for 3 days before baseline testing. Rats were then put under inverted plastic boxes on an elevated mesh floor and allowed to habituate for 30 minutes before the testing. Paw withdrawal thresholds (PWTs) were measured with von Frey filaments (Stoelting, Kiel, WI) in a blinded manner. The ipsilateral hind paw was pressed with one of a series of von Frey filaments with gradually increasing stiffness (2, 4, 6, 8, 10, 15, and 26 g) applied to the plantar surface for 5-6 seconds for each filament. Acute withdrawal, biting, licking, or shaking of the ipsilateral hind limb and vocalization were considered to be positive signs of withdrawal. The behavioral testing was performed blind with respect to the drug administration.

Motor Function Test

In order to assess whether systemic triptolide injection could influence motor function, we performed rotarod tests on rats that had been injected with triptolide but had not had SNL surgery and mechanical allodynia test rats. The protocols were similar to previous reports (16). Rats were placed on the Ugo Basile 7650 Rotarod accelerator treadmill (Ugo Basile, Varese, Italy) set at the minimal speed for training sessions of 1–2 minutes at intervals of 30–60 minutes. Then the rats were placed on to the rotarod at a constant speed of 25 revolutions per minute. As the animal gripped the drum, the accelerator mode was selected on the treadmill. Thereafter, the time was measured from the start of the acceleration period until the rat fell off the drum. Each rat was tested 30 minutes before drug administration as the control performance and was then tested once a day for a week, after 11-day treatments with triptolide. The time that the animal remained on the rotarod was recorded and expressed as a percentage of that animal's own mean control performance.

Western Blot

Rats were anesthetized with pentobarbital (60 mg/kg, intraperitoneal) and were rapidly sacrificed. The left L5 spinal dorsal horn was dissected on dry ice and was then homogenized in SDS sample buffer with a mixture of proteinase and phosphatase inhibitors (Sigma). The crude homogenate was centrifuged at 4°C for 15 minutes at 1,000 gravity. The electrophoresis samples were heated at 99°C for 5 minutes and loaded onto 10% SDS-polyacrylamide gels with standard Laemmli solutions (Bio-Rad Laboratories, CA). The proteins were electroblotted onto a polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore, Billerica, MA). After being blocked in the solution containing Tris-buffered saline with 0.02 % Tween-20 (TBS-T) and 3% nonfat milk for 1 hour, the membrane was incubated overnight with primary antibodies: rabbit anti-phosphorylated c-Jun N-terminal kinase (pJNK, 1:1000; Cell Signaling Technology, Beverly, MA), rabbit anti-JNK (1:1000; Cell Signaling), rabbit anti-phosphorylated extracellular signal-regulated kinase (pERK, 1:1000; Cell Signaling), rabbit anti-ERK (1:1000; Cell Signaling), rabbit anti-phosphorylated p38 (pp38, 1:1000; Cell Signaling), rabbit anti-p38 (1:1000; Cell Signaling), and mouse anti-GAPDH (1:20,000; Millipore). The immunoblots were then reacted with the relative horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit 1:3000, anti-mouse 1:5000; Amersham Pharmacia Biotech Inc., Piscataway, NJ). All

reactions were detected by the enhanced chemiluminescence (ECL) detection method (Amersham) and exposure to film. The same size of square was drawn around each band to measure the density and the background near that band was subtracted. Target protein levels were normalized against GAPDH levels and expressed as relative fold changes compared to the naïve control group.

Immunofluorescent Histochemistry

Rats were perfused with 100 mL of normal saline followed by 500 mL of 0.1 M phosphate buffer (PB, pH 7.3) containing 4% paraformaldehyde. After the perfusion, the L5 spinal cord was removed and postfixed for 2–4 hours and then cryoprotected. Transverse spinal sections (30 µm) were cut in a cryostat, collected in 0.01 M phosphate-buffered saline (PBS, pH 7.3). After being blocked with 2% goat serum, the sections were incubated overnight at 4°C with the primary antibodies: mouse anti-glial fibrillary acidic protein (GFAP) (1:5000; Millipore), mouse anti-NeuN (1:3000; Millipore) or mouse anti-OX-42 (1:500, Serotec, Raleigh, NC). The sections were then incubated for 2 hours at RT with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:800; Molecular Probes). Images were obtained using a confocal laser microscope (FV1000; Olympus, Tokyo, Japan).

The protocol of image analysis was similar to a previous study (6). Five nonadjacent sections from the L5 segments were selected randomly. Images were evaluated by a computer-assisted image analysis program (MetaMorph 6.1, Molecular Devices, Sunnyvale, CA). Image data were collected using the same region and the same size of field within the spinal dorsal horn. The same configuration was used to measure cell areas in all experimental groups. Then the immunoreactivities for GFAP and OX42 within the superficial dorsal horn were averaged across the 5 spinal sections for each experimental group (19).

Real-Time RT-PCR

Rats were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal) and the left L5 spinal dorsal horn was rapidly harvested and total RNA was extracted with Trizol (GIBCO/BRL Life Technologies Inc., Grand Island, NY). Complementary DNA (cDNA) was synthesized with oligo (dT)₁₂₋₁₈ using Superscript™ Reverse Transcriptase for RT-PCR (Invitrogen, Carlsbad, CA). The primers used in the present study are presented in Table 1. Equal amounts of RNA were used to prepare

Table 1. Primers sequence for the rat genes characterized in this experiment.

Genes	Primers	Sequences	Accession number
TNF-alpha	Forward primer	5'-TGATCGGTCCCAACAAGG A-3'	AY427675
	Reverse primer	5'-TGCTTG GTG GTTTGCTACGA-3'	
IL-1beta	Forward primer	5'-TGCTGATGTACCAGTTGGGG-3'	NM031512
	Reverse primer	5'-CTCCATGAGCTTGTACAAG-3'	
MCP-1	Forward primer	5'-CAGATCTCTCTCCTCCACCACTAT-3'	M57441
	Reverse primer	5'-CAGGCAGCAACTGTGAACAAC-3'	
IL-6	Forward primer	5'-GCCCTTCAGGAACAGCTATG-3'	NM012589
	Reverse primer	5'-CAGAATTGCCATTGCACAAC-3'	
GAPDH	Forward primer	5'-CCCCAATGTATCCGTTGTG-3'	NM01008
	Reverse primer	5'-TAGCCCAGGATGCCCTTAGT-3'	

cDNA using the SYBR Premix Ex Taq (Takara, Tokyo, Japan) and analyzed by real-time polymerase chain reaction in a detection system (Applied Biosystems, Foster City, CA). The amplification protocol was: 3 minutes at 95°C, followed by 45 cycles of 10 seconds at 95°C for denaturation and 45 seconds at 60°C for annealing and extension. Target cDNA quantities were estimated from the threshold amplification cycle number (Ct) using Sequence Detection System software (Applied Biosystems). A Δ Ct value was calculated for each sample by subtracting their Ct value from the Ct value for the corresponding GAPDH to normalize the differences in cDNA aliquots. Each cDNA quantity was then calculated with the following formula: 2^{Δ Ct.

Statistics

For behavioral studies, the data of paw withdrawal thresholds passed the normality test, thus were suitable for parametric statistics. The data were analyzed by repeated one-way ANOVA followed by the Student–Newman–Keuls test (multiple groups) or the t test (2 groups) for post hoc analysis. The integrated density from the immunostaining, Western blot and PCR data were analyzed using a 2-way ANOVA with surgery and drug administration as main effects. All the data were presented as mean \pm standard error of the mean; $P < 0.05$ was considered statistically significant in all cases.

RESULTS

Single Treatment of Triptolide on Mechanical Allodynia

To test the effects of a single bolus injection of triptolide on SNL-induced allodynia, we injected triptolide

systemically on post-SNL day 1 when the neuropathic pain was in the induction stage and day 10, when the neuropathic pain was in the maintenance stage. We then observed the behavioral response at 2 hours, 6 hours, 12 hours, 1 day, 3 days, and 7 days after injection. On day 1 in the SNL-vehicle group, PWTs were significantly decreased compared with that of the sham groups. No difference of PWTs could be detected between triptolide and vehicle treatment, at any dosages or at any time points after administration (Fig. 1A). In addition, triptolide treatment in a single bolus injection could not reverse SNL-induced mechanical allodynia on day 10 (Fig. 1B).

Repeated Injection of Triptolide on Mechanical Allodynia

On day 7, SNL rats that received vehicle treatment developed a significant decrease in PWTs, in comparison with sham control animals receiving either triptolide or vehicle (Fig. 2). To determine whether triptolide could prevent the development of mechanical allodynia, we injected triptolide intraperitoneally for 11 days, starting from 3 days before SNL to one week after SNL. After treatment with triptolide, PWTs in the SNL-triptolide group were significantly higher than that of the vehicle control group at each relative time point (Fig. 3A). To investigate whether triptolide would reverse established neuropathic pain, a treatment mode more relevant to a clinical situation, we injected triptolide intraperitoneally on day 10 after SNL. Similar to the prophylactic treatment, the triptolide was also intraperitoneally injected for 11 days, until 20 days after SNL. This treatment effectively reversed SNL-induced mechanical allodynia (Fig. 3B).

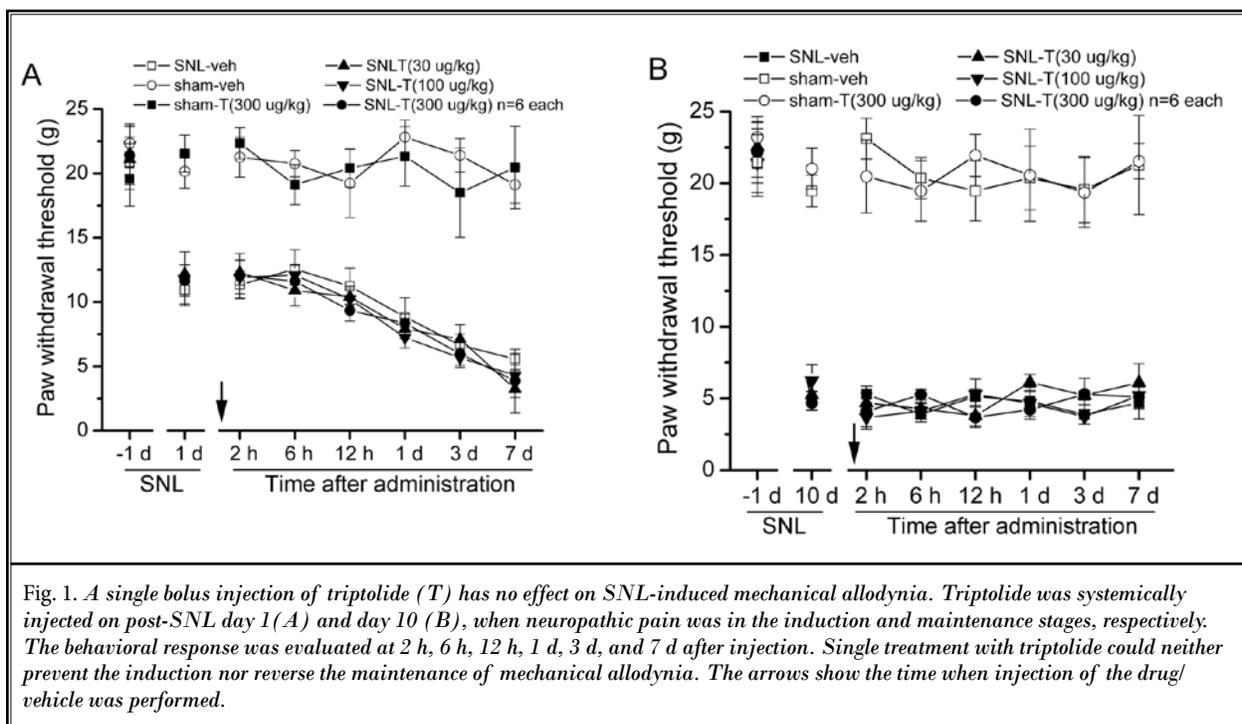


Fig. 1. A single bolus injection of triptolide (T) has no effect on SNL-induced mechanical allodynia. Triptolide was systemically injected on post-SNL day 1 (A) and day 10 (B), when neuropathic pain was in the induction and maintenance stages, respectively. The behavioral response was evaluated at 2 h, 6 h, 12 h, 1 d, 3 d, and 7 d after injection. Single treatment with triptolide could neither prevent the induction nor reverse the maintenance of mechanical allodynia. The arrows show the time when injection of the drug/vehicle was performed.

In both the prophylactic and reversal studies, we observed that the effect of systemic triptolide on neuropathic pain was dose-related. In addition, 300 µg/kg of triptolide could prevent/reverse neuropathic pain for at least one week after the treatment stopped; however, the effect of lower dosages of triptolide (100 µg/kg and 30 µg/kg) could only last for 3-5 days.

To exclude the potential influence of motor deficits on the pain behavioral tests, we performed rotarod testing on 24 rats. Compared with the baseline value, there was no difference in the performance of the rats, either in the vehicle control group or triptolide treatment (30 µg/kg, 100 µg/kg, or 300 µg/kg) group.

Spinal Glial Activation

Triptolide treatment (300 µg/kg) had no effect on GFAP or OX42 expression in sham rats (Fig. 4). On day 7 after SNL, the expression of GFAP and OX42 was significantly increased in SNL-vehicle rats. After treatment with triptolide from SNL day -3 to day 7, GFAP and OX42 levels were remarkably decreased, compared with that of the vehicle control group, although still higher than that of the naïve rats.

On day 20 after SNL, the expression levels of GFAP and OX42 in the SNL-vehicle rats were still significantly

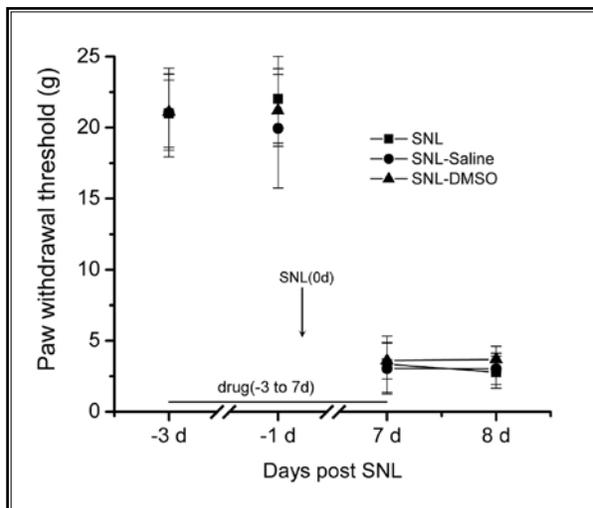


Fig. 2. Neither saline nor DMSO treatment could prevent SNL-induced mechanical allodynia. Eighteen rats were randomly divided into 3 groups: SNL, SNL-saline, and SNL-DMSO. Blank control (nontreatment), saline, or 5% DMSO was given to SNL rats during the period of day -3 (3 d prior to surgery) to day 7 (7 d postsurgery) inclusively (n = 6 rats per treatment group). Behavioral tests showed that no significant difference could be detected in paw withdrawal threshold among these 3 groups.

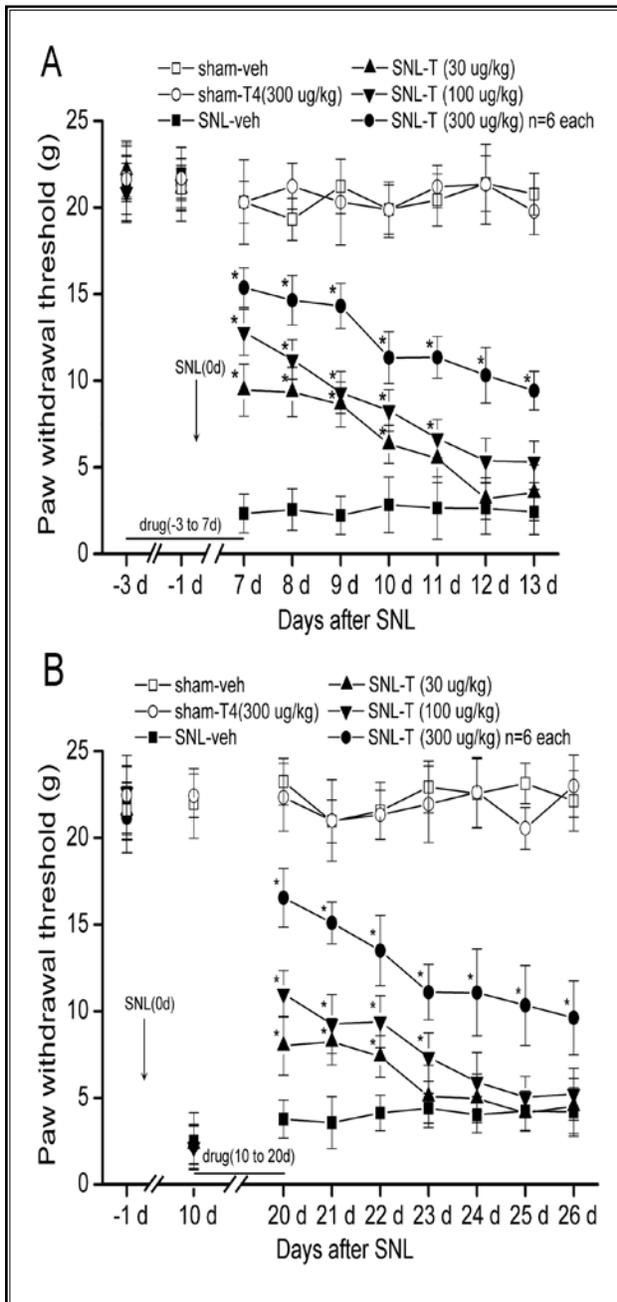


Fig. 3. Repeated injection of triptolide (T) prevents and reverses SNL-induced mechanical allodynia. (A) Triptolide was injected (i.p.) for 11 days, starting 3 d before SNL and until 1 w (A, prophylactic study) or starting on day 10 and will day 20 (B, reversal study) after SNL. After treatment with triptolide, PWTs in the SNL-triptolide group are significantly higher than that of the vehicle control group at each relative time point. In either the prophylactic or reversal studies, the effect of systemic triptolide on neuropathic pain is dose-related. *, $P < 0.05$ compared to the SNL-vehicle group.

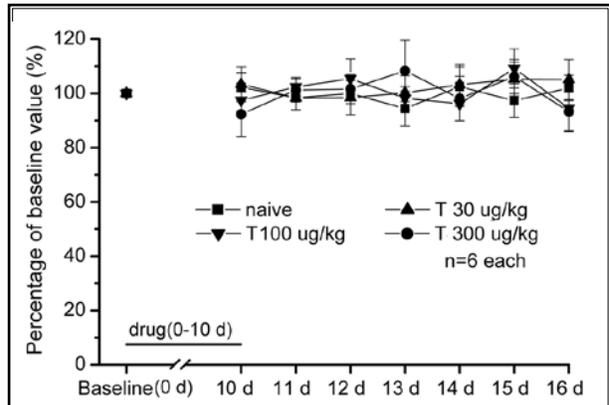
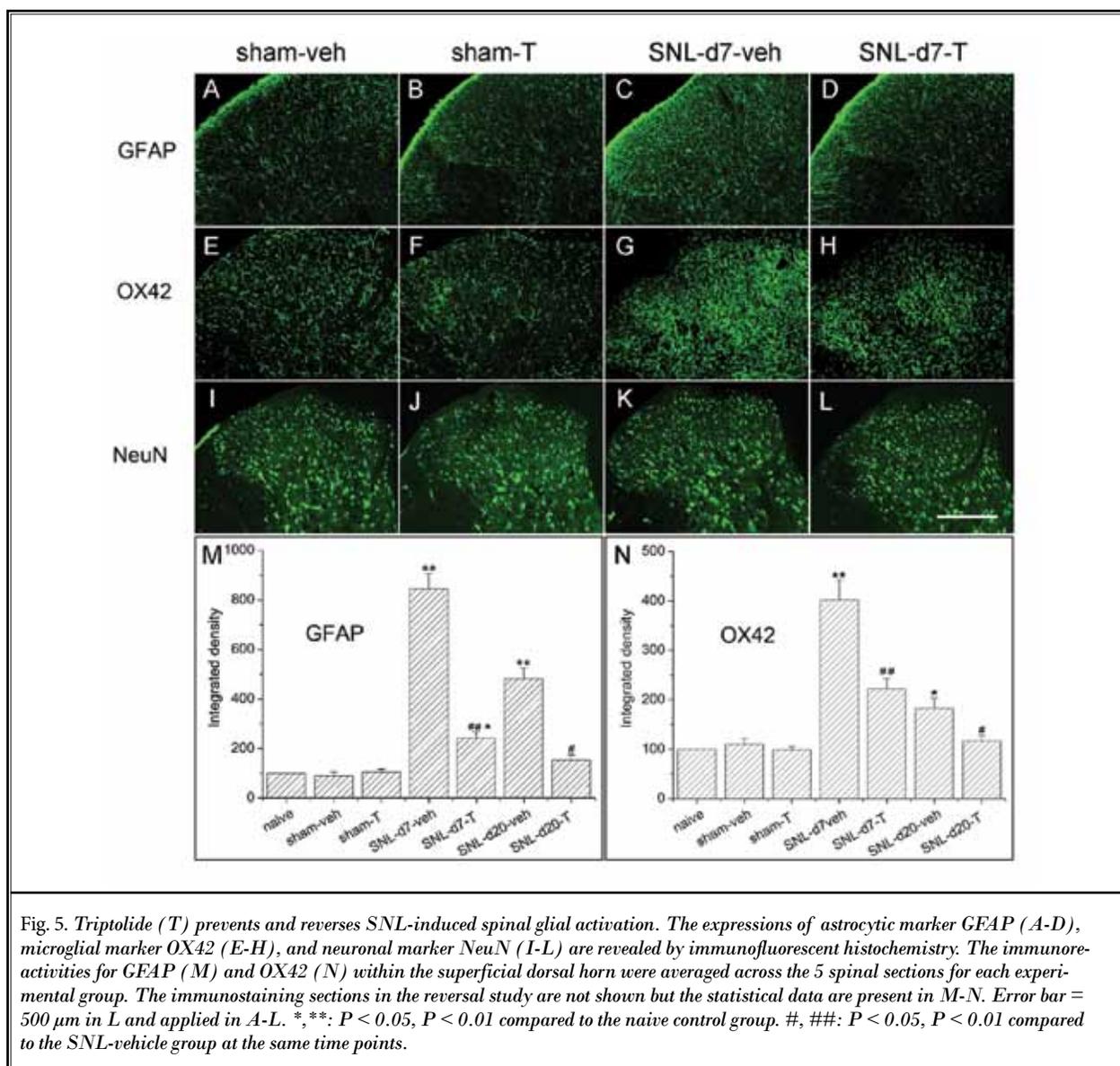


Fig. 4. Systemic injection of Triptolide (T) does not cause motor deficits. After a baseline response was obtained, triptolide (30, 100, and 300 $\mu\text{g}/\text{kg}$) was injected once a day for 11 d and rotarod test was performed once a day for 1 w. Compared with the baseline response, there is no statistical differences were obtained from rotarod test after injection of triptolide.

higher than that of naïve or sham rats, although obviously lower than that of SNL-vehicle rats on day 7 (Fig. 5). After treatment with triptolide from SNL day 10 to day 20, GFAP and OX42 levels were remarkably decreased, compared with that of the vehicle control group (Fig. 5). In addition, the expression of neuronal marker NeuN was not influenced by triptolide treatment in either the prophylactic or reversal study.

Phosphorylation of Spinal MAPKs

The Western blot study showed that in any treatment, the expressions of nonphosphorylated JNK and pJNK-2 were unchanged. Compared with naïve rats, the expression of pJNK-1 was not significantly altered in the sham-vehicle rats (Fig. 6). Triptolide treatment had no effect on pJNK-1 expression in sham rats. On day 7 after SNL, the phosphorylation level of JNK-1 was significantly increased in SNL-vehicle rats (2.72 ± 0.15 folds of naïve control, Fig. 6, $P < 0.05$). After treatment with triptolide from SNL day -3 to day 7, JNK-1 phosphorylation level was remarkably decreased (1.61 ± 0.08 folds, $P < 0.05$), compared with that of the vehicle control group, although still higher than that of the naïve rats. On day 20 after SNL, the expression levels of pJNK-1 in the SNL-vehicle rats (1.95 ± 0.11 folds, $P < 0.05$) were still significantly higher than that of naïve or sham rats, although obviously lower than that of SNL-vehicle rats on day 7. After treatment with triptolide from SNL day 10 to day 20, the pJNK-1 level was remarkably decreased



(1.32 ± 0.06 folds, $P < 0.05$) compared with that of the vehicle control group.

Nonphosphorylated ERK or p38 was not changed in any observed groups in the present study (Fig. 6). The change of pERK was similar to that of pJNK-1. Both pERK-1 and pERK-2 were significantly increased in the SNL-vehicle group on day 7 and day 20 after SNL. Systemic treatment of triptolide, but not the vehicle, effectively prevented and reversed SNL-induced ERK activation (Fig. 6). Another MAPK, p38, was also significantly phosphorylated in the SNL-vehicle group on day 7 but not on day 20 after SNL. Systemic treatment of trip-

tolide effectively prevented SNL-induced up-regulation of pp38 (Fig. 6).

Expression of Inflammatory Mediators

With real-time RT-PCR, we observed that SNL, but not sham surgery, induced significant increases of these inflammatory mediators in the spinal dorsal horn on day 7: IL-6 ($328.3 \pm 43.5\%$ of naive, $P < 0.05$), IL-1beta ($412.6 \pm 46.7\%$, $P < 0.05$), monocyte chemoattractant protein (MCP-1, $343.3 \pm 32.2\%$, $P < 0.05$) and TNF alpha ($243.3 \pm 25.2\%$, $P < 0.05$) (Fig. 7); and on day 20: IL-6 ($282.9 \pm 21.4\%$, $P < 0.05$), IL-1beta ($342.3 \pm 31.7\%$ of

naïve, $P < 0.05$), MCP-1 ($302.3 \pm 25.3\%$, $P < 0.05$) and TNF alpha ($162.3 \pm 13.2\%$, $P < 0.05$). Prophylactic treatment with triptolide significantly prevented IL-6 ($193.5 \pm 19.6\%$ of naïve, $P < 0.05$), IL-1beta ($213.4 \pm 27.8\%$, $P < 0.05$), MCP-1 ($164.4 \pm 28.8\%$ of naïve, $P < 0.05$) and TNF alpha ($143.4 \pm 13.8\%$, $P < 0.05$), compared to the vehicle control group. In addition, reversal treatment with triptolide effectively attenuated the expression of those inflammatory mediators: IL-6 ($173.7 \pm 18.3\%$, $P < 0.05$), IL-1beta ($181.7 \pm 26.1\%$ of naïve, $P < 0.05$), MCP-1 ($151.2 \pm 16.1\%$, $P < 0.05$) and TNF alpha ($113.7 \pm 21.1\%$, $P < 0.05$). These data indicate that systemic treatment with triptolide could prevent and reverse the production of inflammatory mediators in the spinal dorsal horn of neuropathic rats.

DISCUSSION

In the present study, we examined the anti-allodynia effect and the possible mechanism of the Chinese herb extract, triptolide. We made several important observations: repeated injection of triptolide could prevent the induction and block the maintenance of neuropathic pain behavior without influencing basic pain threshold; triptolide significantly inhibits peripheral neuropathy-induced spinal astrocytic and microglial activation; and phosphorylation of MAPKs and synthesis of proinflammatory mediators in the spinal cord of neuropathic rats could be blocked by triptolide. Our data suggest that triptolide may have therapeutic potential in preventing and attenuating neuropathic pain, through inhibiting immune response in the spinal dorsal horn.

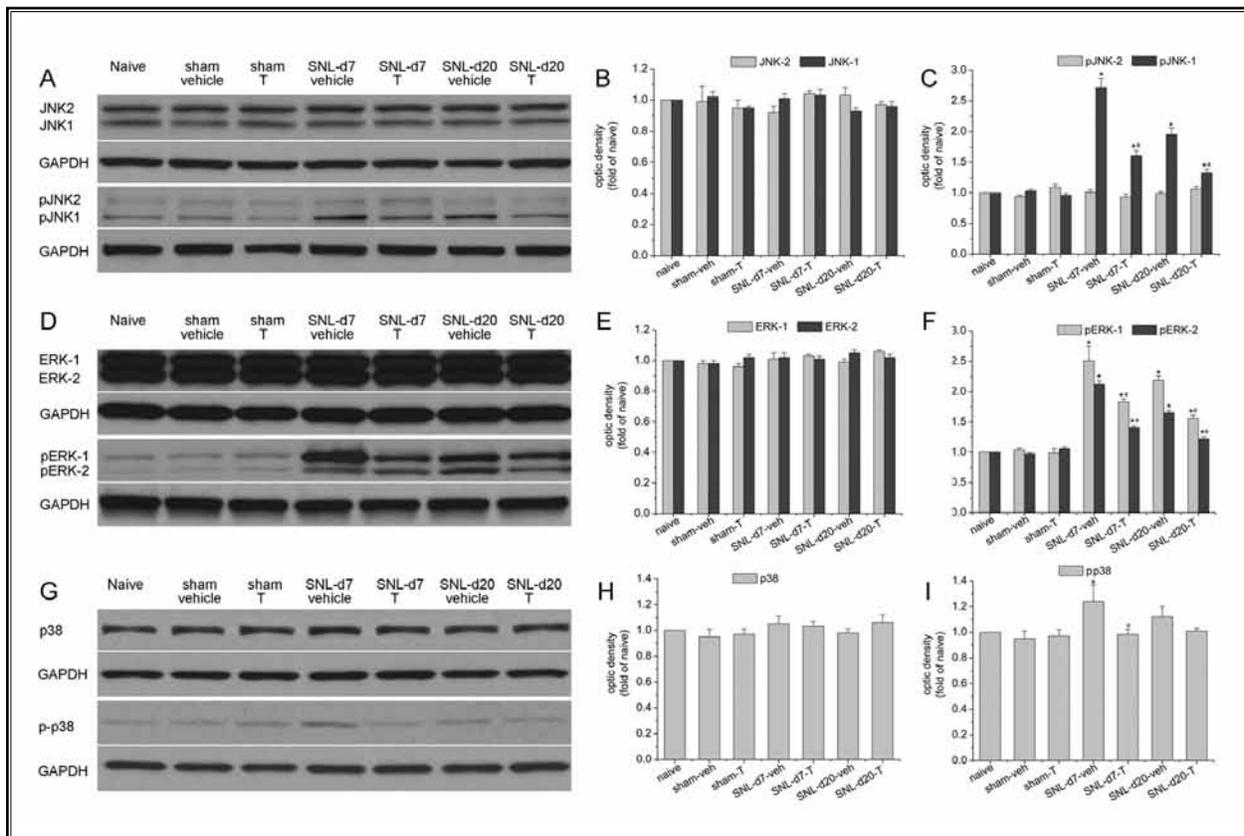


Fig. 6. SNL-induced phosphorylation of MAPKs in the spinal dorsal horn is significantly inhibited by triptolide (T). The expressions of total and phosphorylated (p) form of JNK (A-C), ERK (D-F), and p38 (G-I) are revealed by Western blot analysis. On day 7 after SNL, the phosphorylation levels of JNK-1, ERK1/2, and p38 are significantly increased in SNL-vehicle rats. After treatment with triptolide (300 µg/kg) from SNL day -3 to day 7, those phosphorylation levels are remarkably decreased. On day 20 after SNL, the up-regulated expression of JNK-1, ERK1/2 could also be inhibited by triptolide. Non-phosphorylated MAPKs are not changed after any treatments. * $P < 0.05$ compared to the naïve control group. # $P < 0.05$ compared to the SNL-vehicle group at the same time points.

Accumulating evidence supports the notion that immune response (including activated glial cells and up-regulated expression of cytokines) in the spinal dorsal horn plays essential roles in the induction and maintenance of neuropathic pain (6,20,21). Thus, targeting central immune activation is considered as an effective strategy for treating neuropathic pain. The triptolide extract has a potent anti-inflammatory and immunosuppressive effect and has been used successfully in the treatment of inflammatory diseases (22). Because of its small molecular size and lipophilic properties, triptolide can penetrate the blood-brain

barrier easily, making it a potential drug against inflammatory response in the CNS (23). In the present study, we first reported that repeated systemic administration of triptolide could successfully prevent and reverse neuropathic pain. It is notable that the anti-allodynia effect of triptolide could not be detected after a single bolus injection. The effect of this Chinese herb extract usually has a long time to onset and could also last for a long time. The anti-allodynia effect of triptolide (300 µg/kg) could still be obvious 7 days after administration stopped. Similar phenomena have been observed in other neuroinflammato-

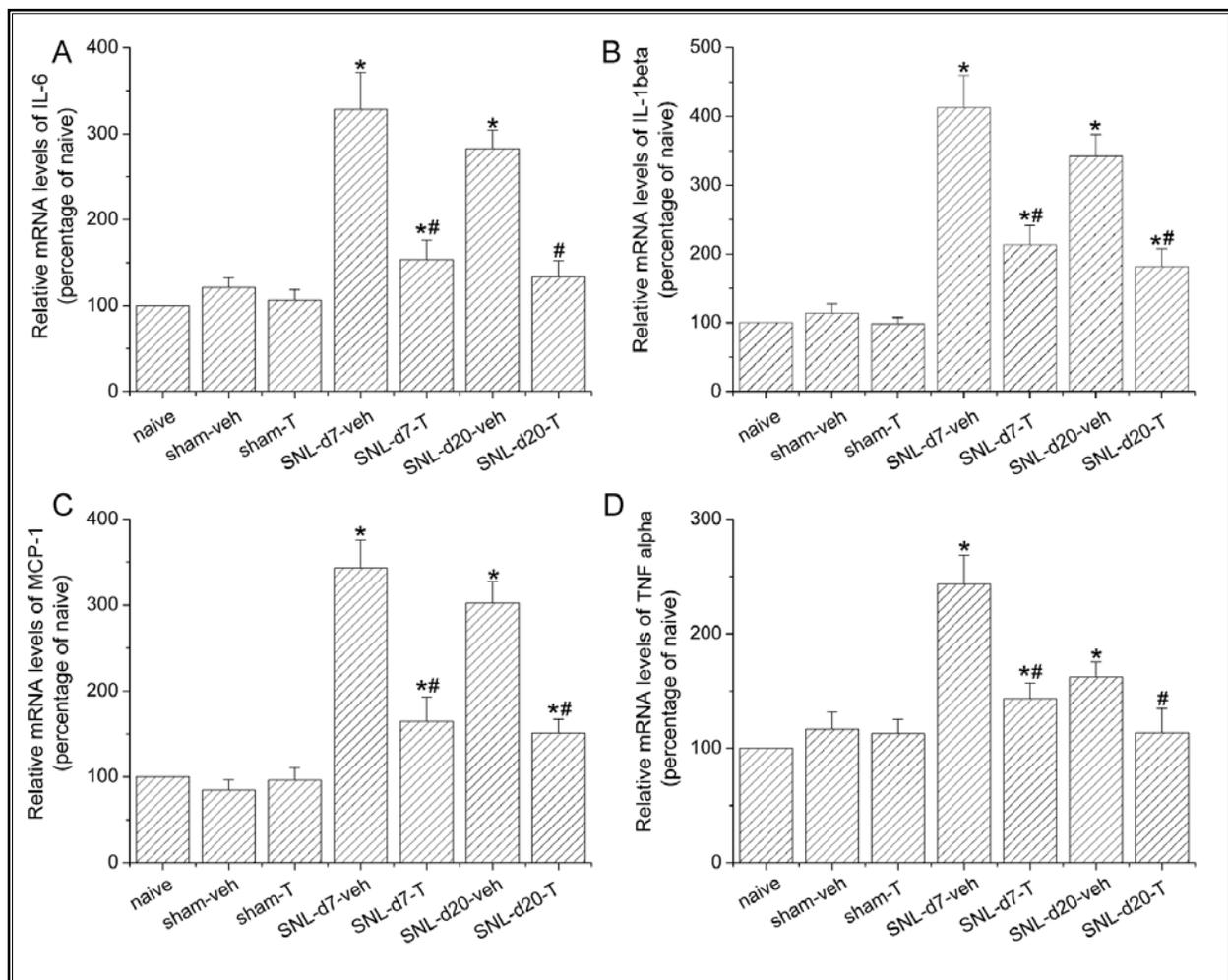


Fig. 7. Triptolide (T) inhibits SNL-induced up-regulated mRNA levels of inflammatory mediators. Synthesis of inflammatory mediators: IL-6, IL-1beta, monocyte chemotactic protein (MCP-1), and TNF alpha are detected by real-time RT-PCR. Data are normalized against GAPDH levels and expressed as a percentage of the naive group. Triptolide significantly prevents and reverses SNL-induced increases of these inflammatory mediators in the spinal dorsal horn. * P < 0.05 compared to the naive control group. # P < 0.05 compared to the SNL-vehicle group at the same time points.

ry diseases. In a rat model of Parkinson disease, the survival rate of dopaminergic neurons could be improved by triptolide treatment for as long as 24 days (13). In a mice model of AA amyloidosis, deposition of amyloid and promoted resorption of splenic amyloid deposits could be observed by treatment with triptolide for 35-105 days (24). Furthermore, after an 11-day systemic treatment with triptolide, astrogliosis and inflammation could be inhibited in a rat model of spinal cord injury (14).

These data suggest that the systemic treatment with triptolide has a strong anti-inflammatory effect, but needs repetitive treatments. Once the effect is obtained, it could last for a long time. We believe this characteristic could make triptolide a long-term treatment for chronic pain. Possibly, in the beginning of the chronic pain treatment, other short-onset analgesics, such as morphine, could be used simultaneously with triptolide. After a few days, triptolide could be the only treatment, thereby avoiding the side effects of long-term morphine treatment. This may be a novel strategy for chronic pain treatment.

We explored the potential underlying mechanism of the anti-allodynia effect of triptolide. It has been reported that triptolide has strong anti-inflammatory activities on microglia (25) and astrocytes (26), which play essential roles in the induction and maintenance of neuropathic pain. It has been shown that triptolide significantly inhibited LPS-activated microglial inflammatory responses (27). In a rat model of spinal cord injury, astrocytic activation could be inhibited by systemic triptolide treatment (14). Interestingly, we observed that the effect of triptolide is confined to glial cells. The numbers and morphology of neurons in the spinal cord are not modulated by triptolide. Previous *in vitro* and *in vivo* studies prove that triptolide could protect neurons from inflammatory insults. The underlying mechanism of this cell type's specific effect is unclear, since the direct target site (such as a specific receptor) of triptolide is still to be determined.

In vitro studies suggest that triptolide could suppress MAPKs phosphorylation (27,28). Activation of MAPKs has been implicated in a number of signaling events that are important for the induction and maintenance of neuropathic pain (29,30). In the SNL model, it is reported that ERK activation could be detected in astrocytes and microglia (31). JNK1 is phosphorylated in spinal astrocytes and p38 is activated in spinal microglia after SNL (18,32). It thus suggests

that the anti-allodynia effect of triptolide is mediated by inhibiting MAPKs activation. MAPKs activation initiates signaling cascades and increases the synthesis of proinflammatory mediators (30). These cytokines could drive central sensitization by increasing excitation in dorsal horn neurons (33). It has been reported that cytokines like IL-1 β released from activated astrocytes could bind to its receptor on neurons and facilitate the NMDAR-related synaptic transmission (5,34). Previous *in vitro* studies have confirmed that triptolide inhibits inflammatory mediators including cytokine (IL-1 β , TNF α , and IL-6) and chemokines (25,35). This anti-inflammatory profile of triptolide is similar to that of the inhibitors of those proinflammatory mediators (34,36). However, triptolide could inhibit a much wider spectrum of these mediators and thus may have a more positive effect, especially in different types of chronic pain where different proinflammatory mediators are involved. Taken together, it is thus believed that MAPKs activation and the following expression of inflammatory cytokines could be the targeting sites of triptolide. Decreased expressions of inflammatory mediators could inhibit the excitatory synaptic transmission during pain processing and thus produce antinociception (Fig. 8). However, MAPKs phosphorylation is initiated by activation of receptors (like purinergic receptors) locating on glial cell membranes (29). It is still to be determined whether and how triptolide could modulate the function of those receptors.

CONCLUSION

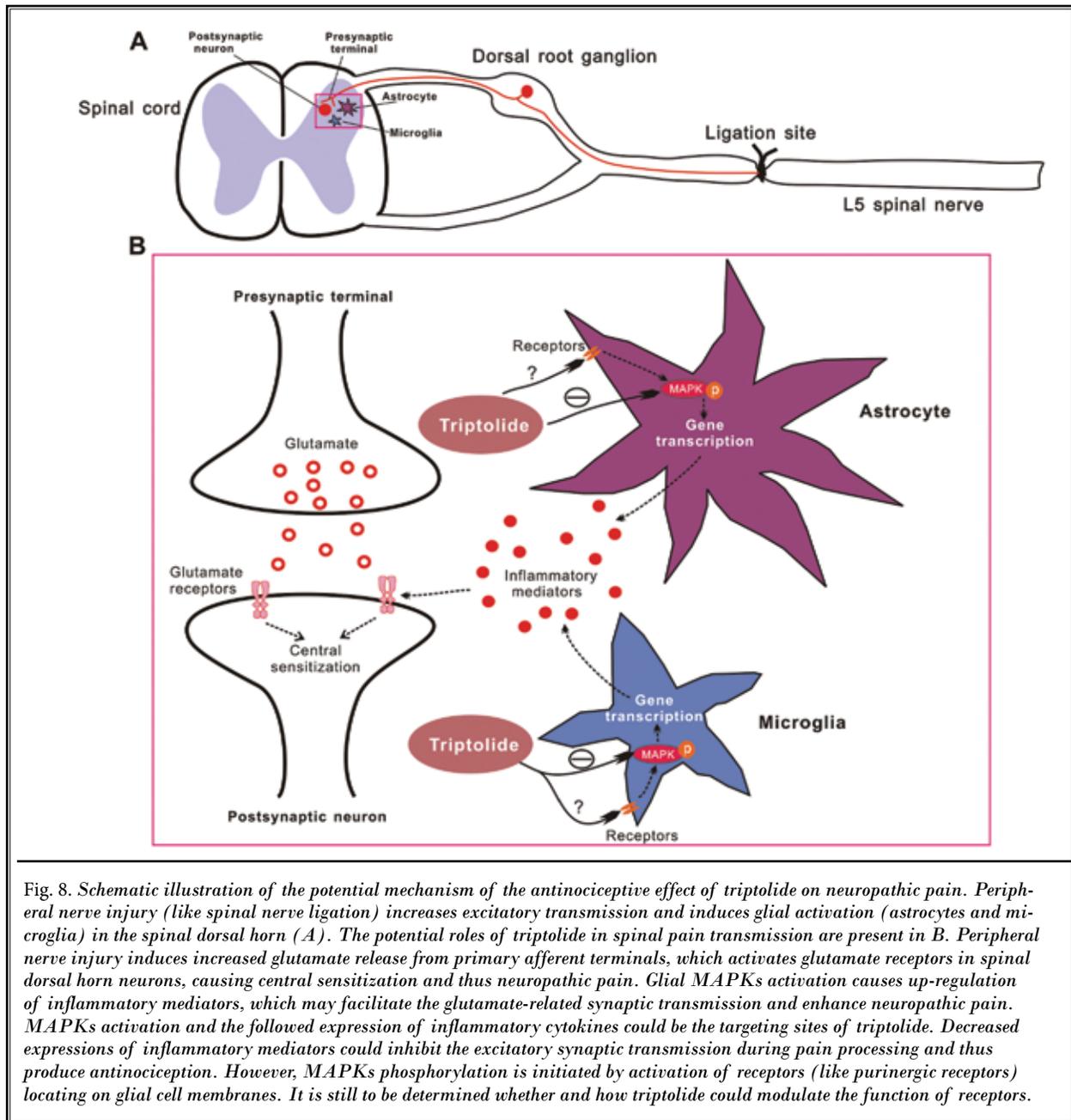
These results demonstrate that triptolide effectively reduced neuropathic pain symptoms via inhibiting central immune response. Comparatively, triptolide has a slow-action but long-term effect in treating neuropathic pain and could inhibit a much wider spectrum of proinflammatory mediators and thus treat various types of neuropathic pain.

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