Original Paper

Depressing Interleukin-1 β Contributed to the Synergistic Effects of Tramadol and Minocycline on Spinal Nerve Ligation-Induced Neuropathic Pain

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Key Words

MAPK · Cytokine · Glia · Analgesics · Allodynia

Abstract

Our previous study indicated that coadministration of tramadol and minocycline exerted synergistic effects on spinal nerve ligation (SNL)-induced neuropathic mechanical allodynia. However, the underlying mechanisms are still unclear. Recent reports indicated that spinal proinflammatory factor interleukin-1 β (IL-1 β) contributed to the development of neuropathic pain and the positive feedback communication between neuron and glia. Therefore, the present research is to confirm whether spinal IL-1 β -related pathway response contributes to the synergistic effects of tramadol and minocycline on SNL-induced neuropathic pain. Real-time RT-PCR demonstrated IL-1 β up-expression in the ipsilateral spinal dorsal horn 3 days after lesion, which could be significantly decreased by tramadol and minocycline coadministration. Immunofluorescence and Western blot indicated that SNL-induced microglial phosphorylated p38 (p-p38) upregulation was also inhibited by tramadol and minocycline coapplication. Meanwhile, intrathecal administration of p38 inhibitor SB203580 markedly alleviated mechanical allodynia whilst reducing IL-1 β and Fos expression induced by SNL. Moreover, intrathecal neutralized antibody of IL-1 β could depress SNL-induced mechanical allodynia and Fos expression. These results suggest that depressing SNL-induced aberrant activation of the spinal dorsal horn IL-1 β -related pathway contributes to the underlying mechanism of the synergistic effects of tramadol and minocycline coadministration on SNL-induced neuropathic mechanical allodynia.

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Introduction

Neuropathic pain is an intractable problem for both clinical treatment and laboratory research. Multiple mechanisms are involved in the development of neuropathic pain, such as superexcited primary afferent, abnormal plasticity in the spinal dorsal horn and aberrant neuron-glia interactions [1–4].

There are many therapeutics given for treating neuropathic pain by the inhibition of spinal nociceptive neuronal activation after nerve injury, such as morphine, gabapentin and tramadol [5, 6]. In addition, there is an abundance of evidence suggesting that spinal glial cell activation is required and is sufficient for neuropathic pain development [7, 8]. Recently, studies have demonstrated that the spinal neuronal and glial ‘cross-talk’ is the most important mechanism underlying the development of neuropathic pain and spinal dorsal plasticity [9, 10]. By releasing neurotransmitters, extracellular signaling molecules, chemokines, cytokines and reuptaking neurotransmitters along the synaptic cleft, glial cells contribute to neuronal excitability and synaptic transmission and thus coordinate activity in neuronal networks [9, 11]. Therefore, inhibiting spinal glial activation is now another option for treating neuropathic pain.

It is known that the clinical concept of a balanced or associative manner proposes to use a combination of analgesics to provide better pain control [12]. Our previous report [13] has suggested that coadministration of tramadol and minocycline exerted synergistic effects on peripheral nerve injury-induced neuropathic pain. However, the underlying mechanisms are unclear.

Studies have suggested that spinal proinflammatory cytokines such as tumor necrosis factor- α (TNF α), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) are responsible for the development of neuropathic pain and the positive feedback communication between neuron and glia [14–17]. In particular, IL-1 β is a potential target in the management of neuropathic pain after injury. A previous study demonstrated that mRNA and the protein level of IL-1 β were rapidly upregulated in the peripheral nerve-injured mouse. Mice lacking IL-1 β showed reduced nociceptive sensitivity (mechanical allodynia) compared with wild-type littermates after injury. Microinjecting recombinant IL-1 β at the site of sciatic nerve injury in IL-1 β -knock-out mice restored mechanical pain thresholds back to levels observed in injured wild-type mice. These results suggest that targeting specific IL-1 β -dependent responses is a key therapeutic target for treating neuropathic pain after peripheral nerve injury [18].

On account of the above, the present research was designed to disclose whether spinal IL-1 β -related pathway response contributes to the synergistic effects of tramadol and minocycline on spinal nerve ligation (SNL)-induced neuropathic pain.

Materials and Methods

Experimental Animals

Male Sprague-Dawley rats (170–190 g) were housed and maintained on a 12:12-hour light/dark cycle under conditions of 22–25°C ambient temperature with food and water available in plastic cages. All experimental procedures received prior approval (No. 11012) from the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi’an, PR China) and were conducted according to the ethical guidelines to investigate experimental pain in conscious animals [19]. Efforts were made to minimize animal suffering and to restrict the number of animals used.

Intrathecal Intubation

Intrathecal intubation was performed by inserting polyethylene tubing to inject the drug directly into the subarachnoid space of the lumbar enlargement. Briefly, a midline incision (3 cm) was made at the back of the rat at the level of the thoracic vertebrae under pentobarbital anesthesia (45 mg/kg, i.p.). A premea-

sured length of polyethylene-10 tubing (inner diameter 0.28 mm, outer diameter 0.61 mm) was passed caudally from the T8 to the L3 level of the spinal cord, with 2 cm of the tubing left exposed in the upper thoracic region. The rats were allowed to recover for 5 days before further use. Only the animals judged as neurologically normal and showing complete paralysis of both hind legs and tail after administration of 2% lidocaine (10 μ l) through the intrathecal intubation were used for the following experiments.

Drug Administration

Tramadol hydrochloride and minocycline hydrochloride (Sigma, St. Louis, Mo., USA) were dissolved and diluted with preservative-free normal saline solution for administration. Normal saline (0.9%) was used as the negative control. The dose for intrathecal administration (tramadol 10 μ g/rat, minocycline 30 μ g/rat or combination tramadol 3.9 μ g/rat + minocycline 27.3 μ g/rat) was based on our previous study [13].

The p38 inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580, 10 μ g/10 μ l; Calbiochem, La Jolla, Calif., USA) and neutralized antibody of IL-1 β (500-p80, 5 μ g/10 μ l; PeproTech, Rocky Hill, N.J., USA) were injected intrathecally, respectively. Drugs and saline (10 μ l each) were injected intrathecally over 30 s, followed by a 10- μ l flush of normal saline.

Spinal Nerve Ligation

To create the SNL model, the left L6 transverse process was first removed to expose the L4 and L5 spinal nerves under pentobarbital anesthesia (45 mg/kg, i.p.). The L5 spinal nerve was then carefully isolated and tightly ligated with 6-0 silk thread [20]. The surgical procedure for the sham group was identical to that of the SNL group, except that the L5 spinal nerve was not ligated.

Nociceptive Behavioral Tests

The animals were acclimatized to the testing environment for 3 days before baseline testing, and were then placed under inverted plastic boxes (30 $\times$ 30 $\times$ 50 cm³) on an elevated mesh floor to allow habituation for 30 min before threshold testing. Room temperature (22–25°C) and humidity remained stable for all experiments. Briefly, a logarithmic series of 8 calibrated Semmes-Weinstein monofilaments (von-Frey hairs; Stoelting, Kiel, Wisc., USA) were applied to the ipsilateral hind paw to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. The log stiffness of the hairs is determined by log₁₀ (milligrams $\times$ 10) [21]. The 8 filaments had the following log-stiffness values (value in milligrams is given in parentheses): 4.17 (1,479 mg), 4.31 (2,041 mg), 4.56 (3,630 mg), 4.74 (5,495 mg), 4.93 (8,511 mg), 5.07 (11,749 mg), 5.18 (15,136 mg) and 5.46 (28,840 mg). The range of monofilaments (1,479–28,840 mg) produced a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity (expressed as log₁₀) [22]. The behavioral responses were used to calculate the 50% paw withdrawal threshold, by fitting a gaussian integral psychometric function using a maximum-likelihood fitting method, as described in detail previously [21, 23]. This fitting method allowed parametric statistical analysis.

Immunofluorescence

Rats were perfused through the ascending aorta with 100 ml 0.9% saline followed by 500 ml 0.1 M phosphate buffer (PB, pH 7.3) that contained 4% paraformaldehyde with pentobarbital (60 mg/kg, i.p.) anesthesia. The L5 spinal segment was subsequently removed and postfixed in the same fixative for 2–4 h and then cryoprotected for 24 h at 4°C in 0.1 M PB containing 30% sucrose. Transverse frozen spinal sections (30 μ m thick) were cut in a cryostat (Leica CM1800; Heidelberg, Germany) and collected serially in three dishes. Each dish contained a complete set of serial sections that were processed for immunofluorescent staining. One of the dishes was selected randomly. The sections in that dish were rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.3) 3 times (10 min each), blocked with 3% donkey serum in 0.01 M PBS that contained 0.3% Triton X-100 for 1 h at room temperature, and then used for immunofluorescent staining.

For double immunofluorescence, sections were incubated with a mixture of two primary antibodies, mouse anti-OX42 (1:500; Abcam, Cambridge, UK) and rabbit anti-p-p38 antibody (1:500; Cell Signaling Technology, Beverly, Mass., USA) overnight at 4°C, followed by a mixture of the two respective secondary antibodies (Alexa 488 donkey anti-rabbit IgG and Alexa 594 donkey anti-mouse IgG, 1:500; Invitrogen, Carlsbad, Calif., USA). The specificity of the staining was tested on the sections in another dish by omission of the primary specific antibodies. No immunoreactive products were found on the sections (data not shown). Confocal images were obtained using a confocal laser microscope (1 μ m thickness of optical section, FluoView FV1000; Olympus, Tokyo, Japan) and digital images were captured with the FV1000.

Table 1. The detailed experimental procedure

Day	Procedure
POD -5	Intrathecal intubation
POD -4	Recovery
POD -3	Recovery and acclimatization
POD -2	Recovery and acclimatization
POD -1	Recovery and acclimatization
POD 0	Baseline of allodynia Drug administration SNL or sham operation
POD 1	Drug administration
POD 2	Drug administration
POD 3	Drug administration Mechanical allodynia test Sacrificed for Western blot, immunofluorescence or RT-PCR study

Western Blot

All animals were sacrificed and the L5 dorsal horns were promptly removed and frozen on dry ice. The spinal dorsal horn was dissected using the open book method [24]. The selected region was homogenized with a hand-held pestle in an SDS sample buffer (10 ml/mg tissue), which contained a cocktail of proteinase and phosphatase inhibitors. The electrophoresis samples were heated at 100 °C for 5 min and loaded onto 10% SDS-polyacrylamide gels with standard Laemmli solutions (Bio-Rad Laboratories, Hercules, Calif., USA). The proteins were electroblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, Mass., USA). The membranes were placed for 1 h in a blocking solution containing Tris-buffered saline with 0.02% Tween (TBS-T) and 5% nonfat dry milk, and incubated for 2 nights at 4 °C under gentle agitation with primary antibodies: rabbit anti-p-p38 antibody (1:1,000, in 5% BSA; Cell Signaling Technology) and mouse anti- β -actin (1:1,000; Sigma). Bound primary antibodies were detected with the anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10,000; Amersham Pharmacia Biotech Inc., Piscataway, N.J., USA). After each step, the immunoblots were rinsed with TBS-T. All reactions were detected by the enhanced chemiluminescence detection method (Amersham). The densities of protein blots were analyzed by using LabWorks software (Ultra-Violet Products, UK). The densities of p-p38- and β -actin-immunoreactive bands were quantified with background subtraction. A square of the same size was drawn around each band to measure the density and the background near that band was subtracted. Since β -actin levels did not change significantly after inflammation and nerve injury [25], the β -actin level was used as a loading control and the p-p38 level was normalized against the β -actin level.

Real-Time RT-PCR

The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and killed. As described previously [26], the ipsilateral L5 spinal dorsal horn was rapidly harvested and total RNA was extracted with Trizol (Gibco/BRL Life Technologies Inc., Grand Island, N.Y., USA). Complementary DNA (cDNA) was synthesized with Oligo (dT)_{12–18} using Superscript™ III Reverse Transcriptase for RT-PCR (Invitrogen). The primers used in the present study are presented in table 1. Equal amounts of RNA (1 μ g) were used to prepare cDNA using the SYBR® Premix Ex Taq™ (Takara, Tokyo, Japan) and analyzed by real-time PCR in a detection system (Applied Biosystems, Foster City, Calif., USA). The amplification protocol was as follows: 3 min at 95 °C, followed by 45 cycles of 10 s at 95 °C for denaturation and 45 s at 60 °C for annealing and extension. All experiments were repeated twice and, in each experiment, PCR reactions were done in triplicate. Target cDNA quantities were estimated from the threshold amplification cycle number (C_t) using Sequence Detection System software (Applied Biosystems). A ΔC_t value was calculated for each sample by subtracting their C_t value from the C_t value for the corresponding GAPDH to normalize the differences in cDNA aliquots. Each cDNA quantity was then calculated with the following formula: $2^{\Delta C_t}$. GAPDH served as an endogenous internal standard control for variations in RT-PCR efficiency.

Rotarod Test

In order to assess whether the drugs used in the present experiment could influence motor function, which might influence the behavioral results, we performed rotarod tests on animals with intrathecal drug administration but without SNL operation and von-Frey test. Rats with no previous exposure to the rotarod test 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 min at intervals of 30–60 min. After this learning period, the animals were placed on the rotarod at a constant speed of 25 rpm. As the animal took a grip of the drum, the accelerator mode was selected on the treadmill, i.e. the rotation rate of the drum was increased linearly at 20 rpm. Thereafter, the time was measured from the start of the acceleration period until the rat fell off the drum. The cutoff time was 30 s. Each rat was tested 30 min before drug administration as control performance and then once a day for 4 days during the drug administration. The time that the animal remained on the rotarod was recorded and expressed as a percentage of that animal's own mean control performance [27].

Quantification and Statistical Analysis

All data were presented as mean $\pm$ SD and collected by researchers blinded to the surgery and reagents used. Data from the von-Frey test were analyzed by repeated-measures ANOVA followed by Fisher's protected least significant difference post hoc comparisons, where appropriate. Data from Western blot and RT-PCR were tested using one-way ANOVA, followed by the least significant difference test. All statistical analyses were performed using SPSS® version 16.0 software (SPSS Inc., Chicago, Ill., USA). $p < 0.05$ was considered statistically significant.

Experimental Procedures

Experimental rats received intrathecal intubation and were allowed to recover for 3–5 days before further use. The baseline value of the behavioral test was carried out on the SNL or sham surgical day prior to the beginning of drug administration. SNL or sham surgical procedure was carried out after drug administration on postoperative day (POD) 0. Drugs were applied for 4 days from POD 0 to POD 3. The behavioral tests were then performed on POD 3 after drug administration. Finally, all rats were sacrificed for immunofluorescence, RT-PCR or Western blot study after the behavioral test on POD 3 (table 1).

Results

Combination of Tramadol and Minocycline Suppressed SNL-Induced Spinal Dorsal Horn IL-1 β and p-p38 MAPK Expression

The expression of spinal dorsal horn IL-1 β was remarkably upregulated in the SNL-saline group compared with the sham-saline group (fig. 1). Intrathecal administration of minocycline 30 μ g (SNL-M30) depressed IL-1 β expression significantly compared with the SNL-saline group (fig. 1). Moreover, tramadol 10 μ g (SNL-T10) could also suppress SNL-induced IL-1 β expression compared with the SNL-saline group (fig. 1). In addition, the combination of tramadol 3.9 μ g with minocycline 27.3 μ g (SNL-T3.9-M27.3) exerted a more powerful inhibiting effect on SNL-induced IL-1 β expression than the administration of tramadol 10 μ g or minocycline 30 μ g alone (fig. 1).

Double immunofluorescent staining with antibodies against OX42 (fig. 2a) and p-p38 (fig. 2b) suggested that all p-p38-positive cells were OX42-positive microglia (fig. 2c) in the ipsilateral spinal dorsal horn 3 days after SNL. The p-p38-positive staining was localized in the cell body, surrounded by the OX42-positive staining (fig. 2d).

The Western blot results suggested that ipsilateral spinal dorsal horn p-p38 expression was remarkably upregulated by SNL compared with the sham-saline group (fig. 2e). Intrathecal minocycline 30 μ g (SNL-M30) or tramadol 10 μ g (SNL-T10) inhibited SNL-induced microglial p-p38 upregulation compared with the SNL-saline group (fig. 2e). Moreover, SNL-induced spinal dorsal horn microglial p-p38 upregulation appeared to be inhibited by the coadministration of tramadol 3.9 μ g with minocycline 27.3 μ g (SNL-T3.9-M27.3) compared with intrathecal administration of minocycline 30 μ g or tramadol 10 μ g alone (fig. 2e).

Fig. 1. Effects of different drug administrations on SNL-induced relative IL-1 β mRNA expression. The y-axis shows the relative IL-1 β mRNA level in the different groups (fold of sham-saline). SNL-induced remarkable IL-1 β up-expression in the SNL-saline group. Intrathecal minocycline 30 μ g (SNL-M30) or tramadol 10 μ g (SNL-T10) could inhibit SNL-induced IL-1 β expression. Coadministration of tramadol 3.9 μ g and minocycline 27.3 μ g (SNL-T3.9-M27.3) also indicated a more powerful suppressing effect on SNL-induced IL-1 β expression than the other groups. * $p < 0.05$ between each group (8 rats in each group).

Intrathecal p38 Inhibitor SB203580 Attenuated SNL-Induced IL-1 β and c-fos mRNA Up-Expression whilst Relieving SNL-Induced Mechanical Allodynia

In order to confirm the effects of intrathecal p38 inhibitor SB203580 on SNL-induced IL-1 β and c-fos mRNA up-expression, SB203580 (10 μ g/10 μ l, twice/day) was applied intrathecally for 4 days from the SNL operation day to POD 3. The expression of IL-1 β and c-fos mRNA was then detected by real-time RT-PCR after the von-Frey test on POD 3.

The von-Frey test suggested that intrathecal p38 inhibitor SB203580 from POD 0 to POD 3 had elevated the SNL-induced mechanical allodynia threshold significantly compared with the SNL-saline group, while the threshold baseline of each group did not show any difference (fig. 3a).

The real-time RT-PCR results indicated that SNL (SNL-saline group) upregulated ipsilateral spinal dorsal horn IL-1 β and c-fos mRNA expression 3 days after lesion, compared with the sham-saline group (fig. 3b, c). Intrathecal administration of p38 inhibitor SB203580 appeared to decrease SNL-induced IL-1 β and c-fos mRNA up-expression on POD 3 compared with the SNL-saline control group (fig. 3b, c). However, the IL-1 β and c-fos mRNA expression of sham operation animals did not change after intrathecal p38 inhibitor SB203580 (sham-SB203580 group).

Intrathecal Neutralized Antibody of IL-1 β Depressed SNL-Induced Neuropathic Mechanical Allodynia and Spinal Dorsal Horn c-fos mRNA Expression

In order to investigate the effects of IL-1 β -neutralized antibody on SNL-induced mechanical allodynia and spinal dorsal horn c-fos mRNA expression, neutralized antibody of IL-1 β (5/10 μ l, twice/day) was injected intrathecally for 4 days from POD 0 to POD 3. The expression of c-fos mRNA was then detected by real-time RT-PCR after the von-Frey test on POD 3.

The mechanical allodynia test with von-Frey showed that the IL-1 β -neutralized antibody alleviated SNL-induced neuropathic pain apparently, compared with that of SNL-saline group. However, IL-1 β -neutralized antibody had no obvious effects on sham-operated animals (fig. 4a).

Real-time RT-PCR demonstrated that intrathecal administration of IL-1 β -neutralized antibody notably suppressed SNL-induced c-fos mRNA up-expression in the ipsilateral spinal

Fig. 2. Effects of different drug administrations on SNL-induced relative p-p38 expression. **a** SNL-induced spinal dorsal horn OX42 expression. **b** SNL-induced spinal dorsal horn p-p38 expression. **c, d** Colocalization of p-p38 and OX42 in the spinal dorsal horn after SNL. **e** SNL-induced spinal dorsal horn p-p38 expression after different intrathecal drugs. * $p < 0.05$ compared with SNL-saline group. # $p < 0.05$ compared with SNL-T3.9-M27.3 group (8 rats in each group). Bars: 50 μ m in **a–c** and 10 μ m in **d**.

dorsal horn, compared with that of the SNL-saline control group (fig. 4b). Nevertheless, *c-fos* mRNA expression of sham operation animals did not show any difference after intrathecal IL-1 β -neutralized antibody.

Effects of Different Drugs on Motor Functions Indicated by Rotarod Test

Nociceptive behavioral results would be influenced by motor dysfunctions. In order to assess whether the drugs used in the present study could impair motor functions, animals

Fig. 3. Different effects of intrathecal p38 inhibitor SB203580. **a** Behavioral test indicated that SB203580 relieved SNL-induced mechanical allodynia threshold significantly. **b, c** Real-time RT-PCR results suggested that intrathecal SB203580 significantly decreased SNL-induced IL-1 β and *c-fos* mRNA up-expression (fold of sham-saline). * $p < 0.05$ compared with sham-saline group. # $p < 0.05$ compared with SNL-saline group (8 rats in each group).

Fig. 4. Effects of intrathecal neutralized antibody of IL-1 β on SNL-induced mechanical allodynia and spinal dorsal horn *c-fos* mRNA expression. **a** Behavioral test showed that IL-1 β -neutralized antibody seemed to alleviate SNL-induced neuropathic pain. **b** Real-time RT-PCR demonstrated that intrathecal administration of IL-1 β -neutralized antibody notably suppressed SNL-induced *c-fos* mRNA up-expression in the ipsilateral spinal dorsal horn (fold of sham-saline). * $p < 0.05$ compared with sham-saline group. # $p < 0.05$ compared with SNL-saline group (8 rats in each group).

Fig. 5. Effects of different drugs on rotarod test. After a baseline response had been obtained, minocycline 30 μ g (M), tramadol 10 μ g (T) and combination tramadol 3.9 μ g with minocycline 27.3 μ g (M + T) were administered intrathecally and the rotarod test was performed once a day for 4 days. In addition, SB203580 10 μ g (SB) and neutralized antibody of IL-1 β 5 μ g (Anti) were also given intrathecally and the rotarod test was performed twice a day for 4 days. Compared with the baseline response, no statistical differences could be detected after drug administration (8 rats in each group).

with intrathecal drug administration but without SNL operation and von-Frey test were assessed with the rotarod test. Drugs applied in the present study (tramadol, minocycline, combination tramadol with minocycline, SB203580, neutralized antibody of IL-1 β) had no obvious effect on the motor performance compared with the baseline level of each animal (fig. 5).

Discussion

A previous report has suggested that coadministration of tramadol and minocycline exerted synergistic effects on peripheral nerve injury-induced neuropathic pain [13]. However, the underlying mechanisms are unclear. Studies have suggested that spinal proinflammatory cytokines such as TNF α , IL-6 and IL-1 β are responsible for the development of neuropathic pain and the positive feedback communication between neuron and glia [14–17]. In accordance with this, the present work demonstrated that IL-1 β was increased in the ipsilateral spinal dorsal horn after peripheral nerve injury, which was significantly decreased by tramadol and minocycline coadministration. In addition, tramadol and minocycline coapplication could inhibit peripheral nerve injury-induced ipsilateral spinal dorsal horn microglial p-p38 upregulation. Intriguingly, intrathecal administration of p38 activation inhibitor SB203580 markedly alleviated mechanical allodynia and reduced IL-1 β and Fos expression induced by peripheral nerve injury. Moreover, the intrathecal neutralized antibody of IL-1 β could depress peripheral nerve injury-induced mechanical allodynia and ipsilateral spinal dorsal horn Fos expression. Therefore, the present study suggests that depressing peripheral nerve injury-induced aberrant activation of the spinal dorsal horn IL-1 β -related pathway contributes to the underlying mechanism of the synergistic effects of tramadol and minocycline coadministration on peripheral nerve injury-induced neuropathic pain.

Tramadol or minocycline has its own ED₅₀. Therefore, the same dose of each drug may exert a different effect. In the present study, the different effect of each drug on the mRNA and protein expression was based on the different mechanism of each drug. The mRNA and protein expression are parallel with the activity of the glial cell, which could be directly

inhibited by minocycline but not by tramadol. The present results are also consistent with previous reports [28–31].

Another study suggested that spinal nerve injury (SNI)-induced mechanical allodynia could be reversed by an intrathecal glial metabolic inhibitor (fluorocitrate). In addition, an intrathecal inhibitor of p38 mitogen-activated kinases prevented SNI-induced mechanical allodynia by inhibiting proinflammatory cytokine IL-1 β production and signaling. Moreover, SNI-induced mechanical allodynia was prevented by intrathecal proinflammatory cytokine antagonists specific for IL-1 β [21]. These results provided the solid evidence for the present study that peripheral nerve injury (SNI or SNL)-induced spinal dorsal horn glial cells, as well as MAPK p38 and proinflammatory cytokine IL-1 β pathway activations, contributed to the development of neuropathic pain. The present study demonstrated that intrathecal tramadol and minocycline depressed SNL-induced spinal dorsal horn microglial p-p38 and IL-1 β production, whilst intrathecal administration of p38 activation inhibitor SB203580 markedly alleviated mechanical allodynia and reduced IL-1 β and Fos expression induced by peripheral nerve injury. Moreover, the intrathecal neutralized antibody of IL-1 β could depress peripheral nerve injury-induced mechanical allodynia and ipsilateral spinal dorsal horn Fos expression. Therefore, suppression of p-p38-dependent IL-1 β production contributed to the synergistic effects of tramadol and minocycline coadministration on spinal microglia and neuron interaction during the development of SNL-induced neuropathic pain.

IL-1 β may be a potential target in the management of neuropathic pain after nerve injury. A previous study demonstrated that mRNA and protein levels of IL-1 β were rapidly upregulated after sciatic nerve injury. Mice lacking in IL-1 β or IL-1-type-1 receptor indicated reduced mechanical allodynia compared with wild-type littermates after nerve injury. Microinjecting recombinant IL-1 β at the site of the sciatic nerve injury in IL-1 β knock-out mice restored mechanical pain thresholds back to levels observed in injured wild-type mice. These results highlighted the fact that targeting specific IL-1 β -dependent responses was a potential therapeutic strategy for the treatment of neuropathic pain after peripheral nerve injury [18].

A previous report observed the effect of electroacupuncture on spinal glia activation and the expression of TNF α and IL-1 β in a nerve injury animal model. It was demonstrated that electroacupuncture notably increased both mechanical and thermal pain thresholds of nerve injury animals. Moreover, electroacupuncture suppressed nerve injury-induced glial activation and up-expression of TNF α and IL-1 β mRNA remarkably [32]. Therefore, nerve injury-induced glial activation and proinflammatory cytokine expression may contribute much to the development of neuropathic pain. The present study demonstrated that the glial inhibitor minocycline combined with tramadol could depress SNL-induced IL-1 β -relative pathway activation, which may be its underlying mechanism for alleviating the SNL-induced mechanical allodynia.

Zhao et al. [33] established an animal model of manganism in male Sprague-Dawley rats. A notable reduction in the number of tyrosine hydroxylase-immunoreactive neurons was observed in the substantia nigra, and the majority of microglial cells were activated. In addition, manganese upregulated IL-1 β protein levels in the substantia nigra. Furthermore, treatment with minocycline, an inhibitor of microglial activation, attenuated microglial activation and mitigated IL-1 β production [33]. These results suggested that activated microglia could influence the function of neurons whilst IL-1 β was involved in the communication of microglia and neuron after lesion. The present results also suggest that spinal dorsal horn IL-1 β contributes to the development of SNL-induced neuropathic pain and cross-talk between microglia and neurons.

Spinal Fos gene expression could be regarded as a marker of activated nociceptive neurons after types of stimulation. Our previous study has demonstrated that spinal Fos expression could be used as a positive marker for activated neurons in the model of SNL-

induced neuropathic pain [34]. The p38 MAPK inhibitor blocked matrix metalloproteinase induction and AP-1 activation in IL-1 β -treated pathological conditions such as osteoarthritis. In addition, the p38 MAPK inhibitor inhibited *c-fos* translocation to the nucleus [35, 36]. The present study demonstrated that tramadol and minocycline coapplication could inhibit peripheral nerve injury-induced ipsilateral spinal dorsal horn microglial p-p38 upregulation. Moreover, intrathecal administration of p38 MAPK activation inhibitor SB203580 markedly alleviated mechanical allodynia and reduced IL-1 β and Fos expression induced by peripheral nerve injury. It could be concluded that the p38/IL-1 β /Fos pathway was involved in the development of SNI-induced neuropathic pain.

A recent study was conducted to investigate the relationship between α_2 -adrenoceptors and the antinociception of tramadol at the spinal level [37]. The results suggested that, with very weak binding affinity for α_2 -adrenoceptors, the antinociception of intrathecal tramadol is partially related to α_2 -adrenoceptors, and its intrathecal antinociception may mainly involve its indirect activation of α_2 -adrenoceptors in the spinal cord. Also, the antinociceptive effect of tramadol was significantly diminished in 5-HT-lesioned mice. Previous data suggested that the descending serotonergic pathways and spinal 5-HT receptors play a crucial role in the antinociceptive and antihyperalgesic effects of tramadol [38, 39]. On the other hand, another recent study observed that the intrathecal injection of minocycline significantly attenuated mechanical allodynia in a rat SNL model [40]. The expression of NMDAR1 was increased in the spinal dorsal horn after SNL, which could be partly inhibited through the intrathecal injection of minocycline. These findings suggested that the attenuation of allodynia in the SNL model following minocycline administration might be associated with the inhibited expression of NMDAR1. Therefore, NMDA-related mechanisms might play an important synergistic role in the minocycline-mediated antinociception when coadministered with tramadol.

It has been observed in our previous report that coadministration of tramadol and minocycline indicated synergistic effects on peripheral nerve injury-induced neuropathic pain [13]. Consequently, the present study suggests that suppressing peripheral nerve injury-induced aberrant activation of the spinal dorsal horn IL-1 β -related pathway contributes to the underlying mechanism of the synergistic effects of tramadol and minocycline coadministration on peripheral nerve injury-induced neuropathic pain. Therefore, these results may provide some new information for clinicians for the treatment of neuropathic pain when combining tramadol and minocycline.

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Disclosure Statement

The authors declare that they have no competing interests.

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