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Original Article

Ligustrazine alleviates spinal cord injury-induced neuropathic pain by inhibiting the TLR4/NF-κB signaling pathway

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Abstract: Objective: To investigate the effects of ligustrazine on neuropathic pain (NPP) in rats with sciatic nerve injury and to provide new scientific insight for broadening the clinical application of ligustrazine. Methods: Human spinal cord cell line STR cells were transfected with TLR4-mimic or mimic negative control (mimic-NC). After transfection, the STR cells were treated with different concentrations of ligustrazine (0, 0.25, 0.5, 1, 2 μm) for 24 h or 48 h. Cell proliferation was detected by MTT assay and colony formation assay. A rat model was further constructed to evaluate mechanical and cold pain sensitivity behaviors by fiber mechanical stimulation and freezing spray. The extracellular fluids of medial prefrontal cortex (mPFC) and central amygdala (CeA) were collected by intracranial dual-site simultaneous microdialysis. The contents of glutamic acid (Glu), aspartate (Asp), glycine (Gly), and γ-aminobutyric acid (GABA) in extracellular fluids were detected by HPLC. Results: Compared to the 0 μm group, ligustrazine concentration at 0.5 μm significantly decreased the relative cell viability of STR cells and promoted the cell apoptosis rate. Ligustrazine at 0.25 μm significantly reduced the colony number of STR cells (all $P<0.05$). Compared to the control group, 1 μM ligustrazine significantly increased the protein expression of Bax and cleaved caspase 3 in STR cells but decreased the protein expression of Bcl-2 (all $P<0.001$). Compared to the control group, 2 μM ligustrazine treatments significantly reduced the protein levels of TLR4 and p-Akt in STR cells (all $P<0.001$). However, 2 μM ligustrazine treatments did not change the protein expression of Akt ($P>0.05$). Compared to the control group, the level of TLR4 in STR cells transfected with TLR4-mimic was significantly increased ($P<0.001$). Compared to the control group, transfection of TLR4-mimic reversed the anti-proliferative and pro-apoptotic effects of ligustrazine on STR cells (all $P<0.001$). Conclusion: The analgesic effect of Ligustrazine on neuropathic pain caused by spinal cord injury may be related to its inhibition of the release of excitatory amino acid transmitters Glu and Gly through the TLR4/NF-κB pathway, regulation of the dynamic balance of excitatory and inhibitory amino acid neurotransmitters, and alleviation of the central sensitization effect caused by the excitotoxicity of Glu.

Keywords: Ligustrazine, TLR4/NF-κB signaling pathway, apoptosis, neuropathic pain, Bax, p-Akt

Introduction

Neuropathic pain (NPP) is a chronic intractable pain commonly seen in clinics, characterized primarily by spontaneous pain and hyperalgesia [1]. It can be triggered by various factors, including trauma, compression, inflammation, metabolic disorders, and abnormal gene expression [2]. The prevalence of NPP is as high as 6% to 8% in the general population and as high as 20% to 24% in patients with diabetes [3]. Among patients aged 50 or older with herpes zoster, 25% to 50% may suffer from postherpetic neuralgia three months post-recovery [4]. The long course of NPP seriously affects patients' quality of life, emotional well-being and social functioning, making NPP a focus of basic and clinical research. Recent studies highlight the therapeutic potential of ligustrazine, a compound known for its spasmolytic properties, ability to reduce vascular resistance, and strong inhibitory effects on platelet

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aggregation. Ligustrazine facilitates the depolymerization of aggregated platelets, leading to decreased whole blood viscosity. Moreover, it enhances coronary flow, optimizes myocardial oxygen supply, and diminishes myocardial oxygen consumption. These effects render ligustrazine an effective treatment option for ischemic cerebrovascular conditions, coronary heart disease (CHD), migraines, and severe cases of pulmonary heart disease [5-7].

The pathogenesis of NPP is multifaceted, encompassing both peripheral and central mechanisms. Peripheral mechanisms involve changes in the microenvironment surrounding the free nerve endings, ectopic discharge of peripheral nerve damaged areas or dorsal root ganglion neurons, increased expression of adrenergic alpha receptors and sympathetic sprouting [8]. The central mechanisms include sensitization of nociceptive neurons in the dorsal horn of the spinal cord, activation of glial cells, long-term enhancement of synaptic transmission efficiency, high central sensitization of the brain, and activation of the central descending facilitation system. Increasingly, researchers are recognizing that central sensitization plays a pivotal role in the onset and persistence of NPP, possibly surpassing the effect of peripheral sensitization [9].

Amino acid transmitters play a critical role in the central nervous system (CNS), particularly in relation to pain processing. Nociceptive stimuli reach the dorsal horn of the spinal cord through fibers A and C [10]. In this way, synapses and glial cells release plenty of excitatory amino acids, including glutamate (Glu) and aspartic acid (Asp), while the release of inhibitory amino acids such as glycine (Gly) and γ-aminobutyric acid (GABA) decreases Glu, notably concentrated in the gray matter of the cerebral cortex, hippocampus, and spinal cord, has been categorized into ionic and metabolic receptors, according to electrophysiological and pharmacological studies [11-13]. The ionic type of Glu receptors includes N-methyl-D-aspartic acid (NMDA) receptor, whereas Metabotropic glutamate receptor (mGluR) is further divided into three categories: mGluR 1/5, mGluR 2/3 and mGluR 4/6/7/8. Glu plays an essential role in the pathogenesis of pain by activating excitotoxicity produced by its receptors and inhibiting oxidative toxicity produced by Glu/cystine transporters on cell membranes. The anterior cingulate cortex positively affects aversive and fearful behaviors caused by nerve injury in animals. Electrical stimulation of this area leads to increased self-harm behavior in animals, a response possibly linked to pain memory activation. In addition, the amygdala plays a vital role in the perception of NPP, especially emotional pain [14]. Current research on the pathogenesis of NPP at home and abroad mainly focuses on the spinal cord. However, this study shifts the spotlight to amygdala and medial prefrontal cortex, critical regions of pain signal transmission and perception in the spinal cord [15].

Many studies have proven that the astrocytes in the dorsal horn of the spinal cord express TLR4 (Toll-like receptor 4), which is involved in chronic pain caused by spinal cord injury (SCI) and diabetic peripheral neuropathy. In SCI rats, a reduction in thermal withdrawal latency (TWL) was observed alongside a notable increase in the expression of TLR4 and MyD88 in the spinal cord, leading to neuralgia. In vivo, mRNA expression of TLR4 and CD14 was up-regulated in the spinal cord four hours post-spinal nerve L5 transection, with CD14 expression peaking at four days after spinal nerve transection, and TLR4 continuing to increase until 28 days after surgery. In rodents with spinal nerve L5 transection (L5Tx), CD14 knockout mice showed a significant reduction in abnormal mechanical pain and thermal hyperalgesia, further proving that CD14 serves as an auxiliary molecule of the TLR4 signaling pathway.

Based on the previous work, this study adopted a rat model of sciatic nerve injury, which aligns closely with the pathological mechanism of NPP and offers stability, to observe the effects of ligustrazine on NPP using behavioral analysis techniques [16-18]. The impact of ligustrazine on TLR4 and the central nucleus of the amygdaloid was observed by intracranial dual-probe microdialysis combined with high-performance liquid-fluorescence assay. This approach is intended to investigate the analgesic mechanism of ligustrazine and to provide a new scientific basis for broadening its clinical application.

Materials and methods

Cell culture

Human spinal cord cell line (STR cells) was purchased from ATCC (USA). The cultures were cul-

tured in RPMI1640 medium (Invitrogen, USA) containing 10% fetal bovine serum, 100 u/mL penicillin, and 100 μg/mL streptomycin at 37°C 5% CO_2.

Cell transfection and ligustrazine treatment

TLR4-mimic (5'-CAGGTGTAGTTTGGCGCTGTCGGCTGTCGACATG-3') and Mimic negative control (5'-ATCTTGTGGTCTTAGGCGGAAACGCTGTCGACTTC-3') were purchased from Shanghai Jima Pharmaceutical Technology Co., LTD. 5 × 10^5 cells were seeded into a 96-well plate to achieve 70% confluence. 1 μl Lipofectamine 2000 (Invitrogen, USA) was diluted with 50 μl of antibiotic-free RPMI1640. 8 pM of TLR4-mimic and mimic-NC were prepared in 50 μl of antibiotic-free RPMI1640 medium. Subsequently, TLR4-mimic or mimic-NC was mixed with an equal volume of Lipofectamine 2000 diluent. The resulting 100 μl of the mixture was added to cells for 6 hours, and then the culture medium was changed to a complete medium for 48 h. Control cells were cultured normally. In addition, after cell transfection, STR cells were treated with different concentrations of ligustrazine (0, 0.25, 0.5, 1, and 2 μM) for 24 or 48 h.

Cell viability assay

Following the treatment with ligustrazine, 20 μl tetrazolium salt (MTT, Sigma Company, USA, 5 mg/mL) was added to each well, and the cells were incubated at 37°C for 4 h. 150 μL dimethyl sulfoxide (DMSO) was added to each well, and the plates were gently shaken for 10 min.

Colony formation experiments

Cells were seeded in 6-well plates at 500 cells/well and cultured in RPMI1640 medium containing different concentrations (0, 0.25, 0.5, 1, 2 μm) of ligustrazine (Tetramethylpyrazine, TMP), 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2 for two weeks. Then, the samples were fixed with 90% ethanol for 30 min and stained with 0.1% crystal violet. The number of colonies of more than 50 cells was counted.

Determination of cell apoptosis

Apoptosis was analyzed by Annexin V-FITC and propidium iodide (PI) staining with kits purchased from Biyuntian Biotechnology Institute. After cells were treated with ligustrazine, cells were harvested and washed three times with ice cold PBS. The cell suspensions were mixed with Annexin V-FITC and PI staining solutions and incubated for a period of time. Flow cytometry was used to analyze cell apoptosis. The stained cell suspension was injected into a flow cytometer, and the cells could be divided into different subsets of apoptotic, necrotic, and viable cells based on the fluorescence signals of Annexin V-FITC and PI.

PCR

Total RNA was extracted from the sample by Trizol (ThermoFisher) and then reverse-transcribed into complementary DNA (cDNA) by HiScript II One Step RT-PCR Kit (P611-01, Vazyme). The specific primer sequences used in the analysis were as follows: For TLR4, the forward primer sequence is 5'-CTGGTGTAGTCTGAGGCGGTAAGGCAGTCAACATC-3' and the reverse primer sequence is 5'-CCGGGTAGACTTAAACGTACTATTC-3'. For NF-κB, the forward primer sequence is 5'-AGTTGGTGAGGTATGGGCCCTCGTACACA-3' and the reverse primer sequence is 5'-AGCTGTGTAAGCATCGGCGGCTCCA-3'. For β-actin, used as a reference gene, the forward primer sequence is 5'-CGGCGTCAACAGAACTGGTA-3' and the reverse primer sequence is 5'-TATCTCGAACGTTGGGTACCCT-3'. TLR4, NF-κB and BNIP2 levels were normalized to β-actin, and the relative expression levels were determined by the $2^{-\Delta\Delta Ct}$ method.

Western blot

The cells were lysed in RIPA lysis buffer (Jiangsu Kaiji Biotechnology Co., LTD.). First, the protein sample was separated by SDS-PAGE electrophoresis (Bio-Rad, USA) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck & Co., USA). Next, the membrane was incubated with specific primary antibodies: Bax (Proteintech, Cat No. 50599-2-Ig, 1:2000), Bcl-2 (Proteintech, Cat No. 26593-1-AP, 1:1000), cleaved caspase 3 (Proteintech, Cat No. 25128-1-AP, 1:1000), NF-κB (Proteintech, Cat No. 66535-1-Ig, 1:1000), p-Akt (Proteintech, Cat No. 66444-1-Ig, 1:1000), Akt (Proteintech, Cat No. 60203-2-Ig, 1:1000), and β-actin (Proteintech, Cat No. 81115-1-RR, 1:1000). Then, the membrane was incubated with HRP-labeled rabbit secondary antibodies (Abcam, USA) at a 1:500 dilution for one hour

at room temperature. Image J was used for protein band quantification.

Animal model

Specified pathogen-free (SPF) C57BL/6 mice, male, 6-8 weeks old, were purchased from Beijing Vitong Lihua Laboratory Animal Co., LTD., China. The mice were raised under relatively sterile conditions, constant temperature and humidity, ensuring an environment free from specific pathogens. The bedding materials were changed regularly, and mice had unrestricted access to autoclaved water and feed. Mice were sacrificed by cervical dislocation. The Ethics Committee of First Affiliated Hospital to Heilongjiang University of Traditional Chinese Medicine approved this experiment.

Mechanical stimulation and cryospray of rats are common tests of pain sensitivity. Mechanical Sensitivity testing: The mechanical sensitivity of the rats was tested using von Frey spines. In this test, the rat's skin was stimulated using a series of fine burrs of different diameters, and the rat's response was observed to assess its mechanical nociceptive threshold. Cold Pain Sensitivity Test: Rats were tested for cold pain sensitivity using cryospray or cold plates. Cold pain sensitivity was assessed by observing the response of rats by spraying the soles of their feet with cryospray or by placing the soles of their feet on a precooled plate.

Preparation of rat model of sciatic nerve injury

Male adult rats were randomly assigned to the following groups: 1. Sham group: the control group where rats underwent a similar surgical procedure without actual nerve damage; 2. Model group: rats underwent sciatic nerve injury surgery but did not receive any other treatment; 3. Model+Lig-VPLNT group: rats received Ligustrazine oral treatment after sciatic nerve injury; 4. Model+Lig-it group: rats received intrathecal injection therapy after sciatic nerve injury; 5. Model+Lig-iv group: rats received intravenous injection therapy after sciatic nerve injury.

Except for the sham group, all other groups of rats had their sciatic nerves compressed using a hemostatic forceps model. At a point 0.5 cm below the femoral condyle, parallel to the course of the sciatic nerve, a 1.0-1.5 cm incision was made, bluntly separated to expose the piriformis muscle, and the left sciatic nerve was isolated. All groups used the same-sized hemostatic forceps to vertically clamp the sciatic nerve trunk, clamping three times for 10 seconds each, with a 10-second interval. The width of the nerve trunk compression injury was approximately 4 mm.

In this study, the mechanical withdrawal reflexes and cold pain sensitivity of rats were observed using mechanical stimulation and the cold spray method. The rats were anesthetized with Pentobarbital (50 mg/kg) to ensure that they did not feel any pain during the experiment. For the mechanical stimulation, a von Frey filament was applied to the dorsum of the rat's hind paw, and the pressure was increased gradually until a withdrawal reflex was observed. The threshold pressure required to elicit the withdrawal reflex was recorded. For assessing cold pain sensitivity, a cold spray consisting of a blend of ethyl chloride and dichlorodifluoromethane, which achieves a temperature of around -30°C, was applied to the rat's hind paw for 3 seconds. The duration until the rat licking or raising its back foot in response to the cold spray was recorded as a measure of complex pain sensitivity.

Determination of Glu, Asp, Gly, and GABA in rat brain dialysate by HPLC

The LC-MS/MS analysis was performed on an AB SCIEX Triple QuadTM 4500MD LC-MS/MS system (ABsciex, Toronto, Canada). A JasperTM HPLC equipped with a CORTECS T3 column (2.1 mm × 100 mm, 2.7 μm; Waters) was employed for chromatographic separation at 40°C. 0.1% formic acid in purified water and 0.1% formic acid in ANC were used in the mobile phase A and the mobile phase B, respectively. The separation was achieved with the following gradient program at a flow rate of 0.35 mL/min: 0.00 min, 15% B; 0.50 min, 15% B; 1.00 min, 35% B; 3.00 min, 60% B; 4.00 min, 80% B; 4.10 min, 95% B; 4.60 min, 95% B; 4.70 min, 15% B and return to initial conditions until 5.8 min. For each sample, the total run time was 5.8 min and the injection volume was 1 μL. The 50% MeOH solution (50:50, MeOH - water, v/v) was used to clean the autosampler syringe between injections. The mass spectrometer (MS) detection was operated with

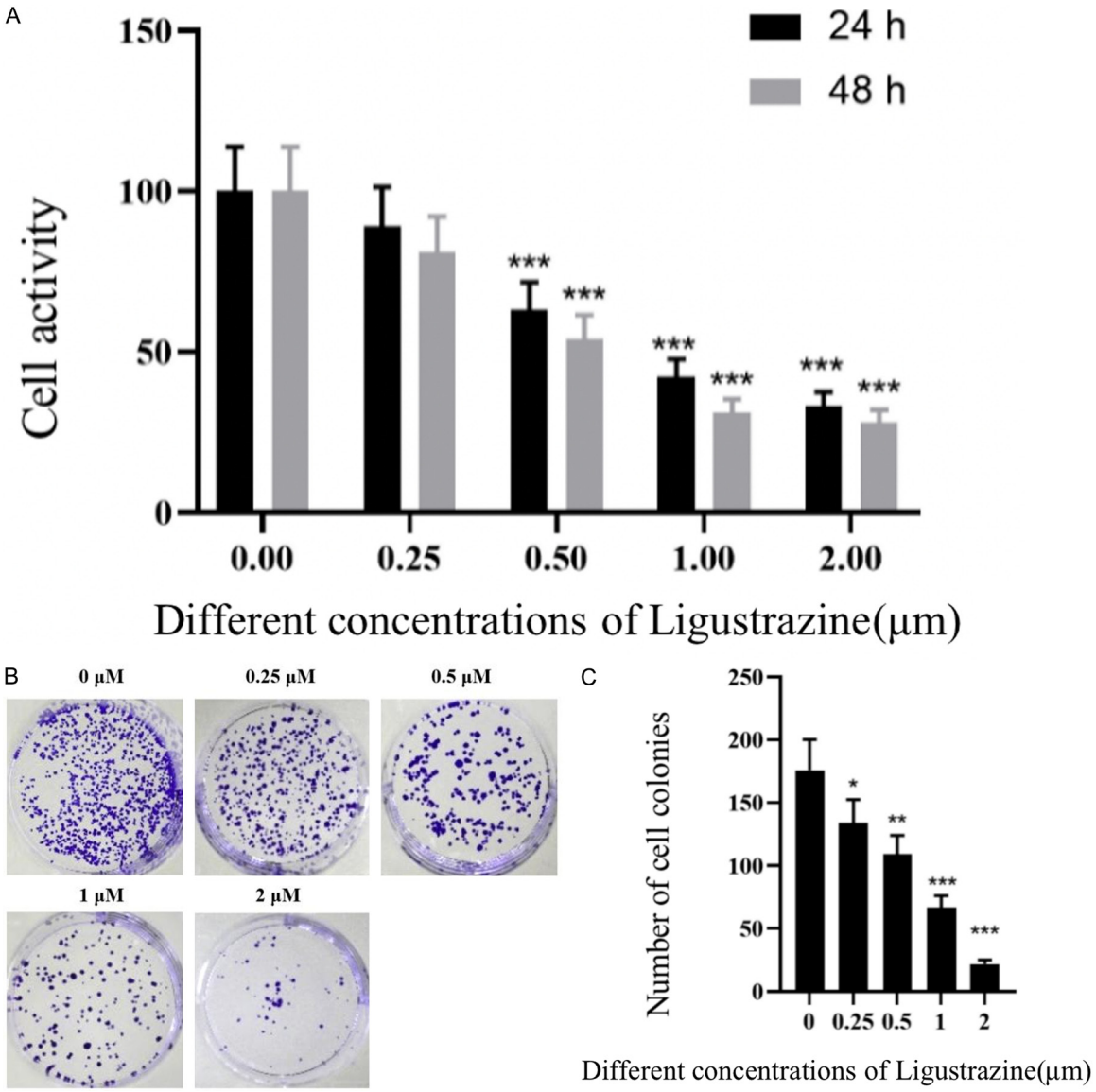

Figure 1. Ligustrazine inhibits the proliferation of cells. Note: (A) MTT method to detect the relative cell viability of cells treated with different concentrations of Ligustrazine (0, 0.25, 0.5, 1, 2 μM) after 24 h or 48 h; (B and C) Colony formation experiments to evaluate the proliferation of cells treated with different concentrations of Ligustrazine (0, 0.25, 0.5, 1, 2 μM) after 24 h or 48 h. Compared to 0 μM, *P<0.05, **P<0.01, ***P<0.001.

multiple reaction monitoring (MRM) in the positive mode. MS parameters were set as follows: ion spray voltage, 5500 V; heated nebulizer temperature, 550°C; curtain gas (CUR), 35 psi; collision gas (CAD), 7 psi; ion source gas 1 (GS1/GS2), 55 psi. All data were collected and analyzed with Analyst MD 1.6.3 and MultiQuant MD 3.0.2.

Immunofluorescence

Cells were fixed using formaldehyde to stabilize the cellular structure and fix the location of the target protein. The permeation agent Triton X-100 was used to penetrate the cell membrane so that the fluorescent dye could enter the cell. Nonspecific binding sites were blocked using bovine serum proteins to reduce background signals. An antibody that binds specifically to the protein to be detected is added so that it reacts with the target protein. Subsequently, fluorescently labeled secondary antibody was added to introduce the fluorescent label into the cells to achieve the labeling of the target protein. Cells were washed with buffer to remove unbound I

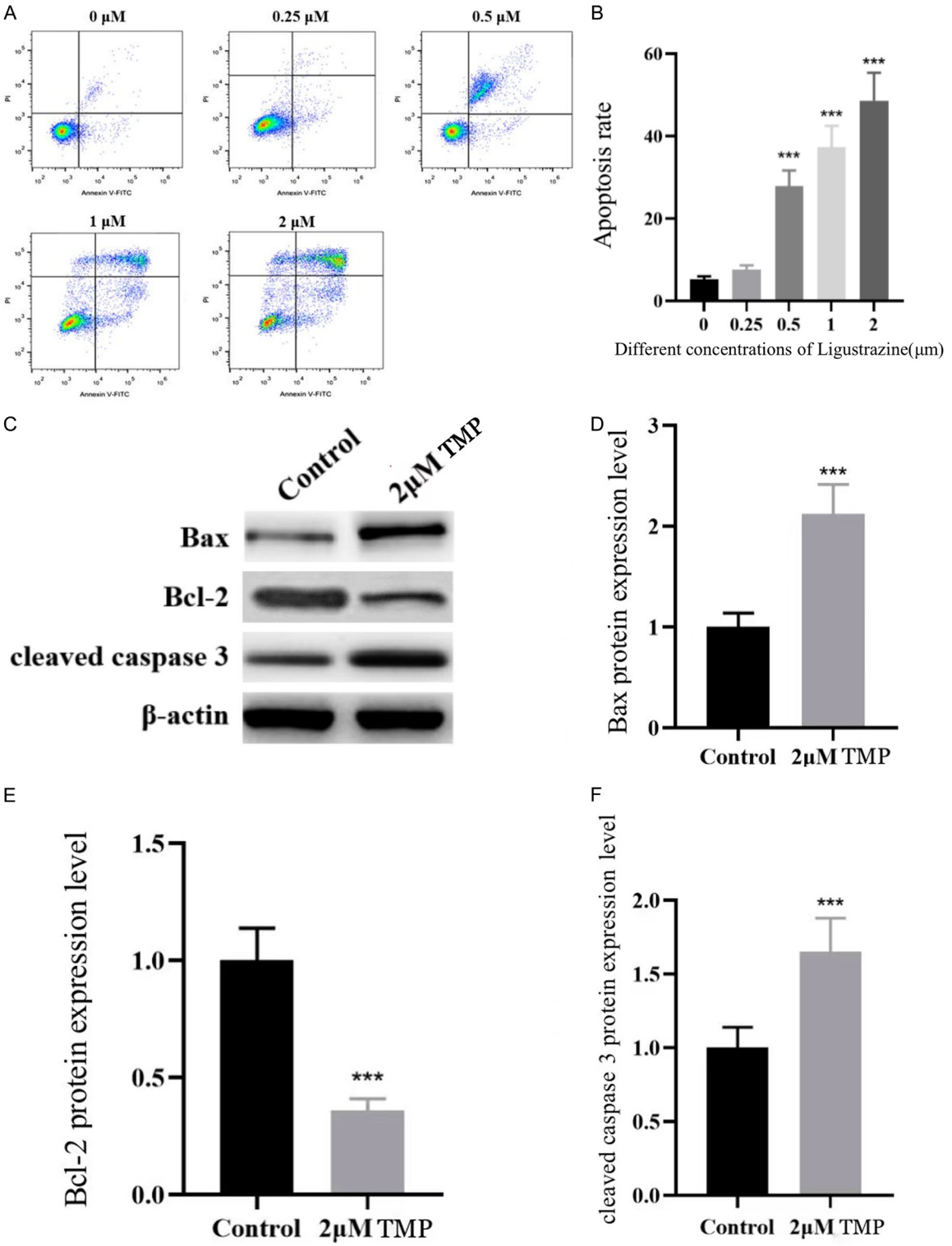

Figure 2. Ligustrazine promotes cell apoptosis. Note: (A and B) Flow cytometry to detect the apoptosis rate of cells treated with two μM of Ligustrazine for 48 h; (C-F) Western blot was used to detect the protein expression of Bax, Bcl-2 and cleaved caspase 3 in cells treated with two μM of Ligustrazine for 48 h. Compared to the control, ***P<0.001.

and II antibodies and reduce background signals.

The cell fluorescence signal was observed under a fluorescence microscope to detect the

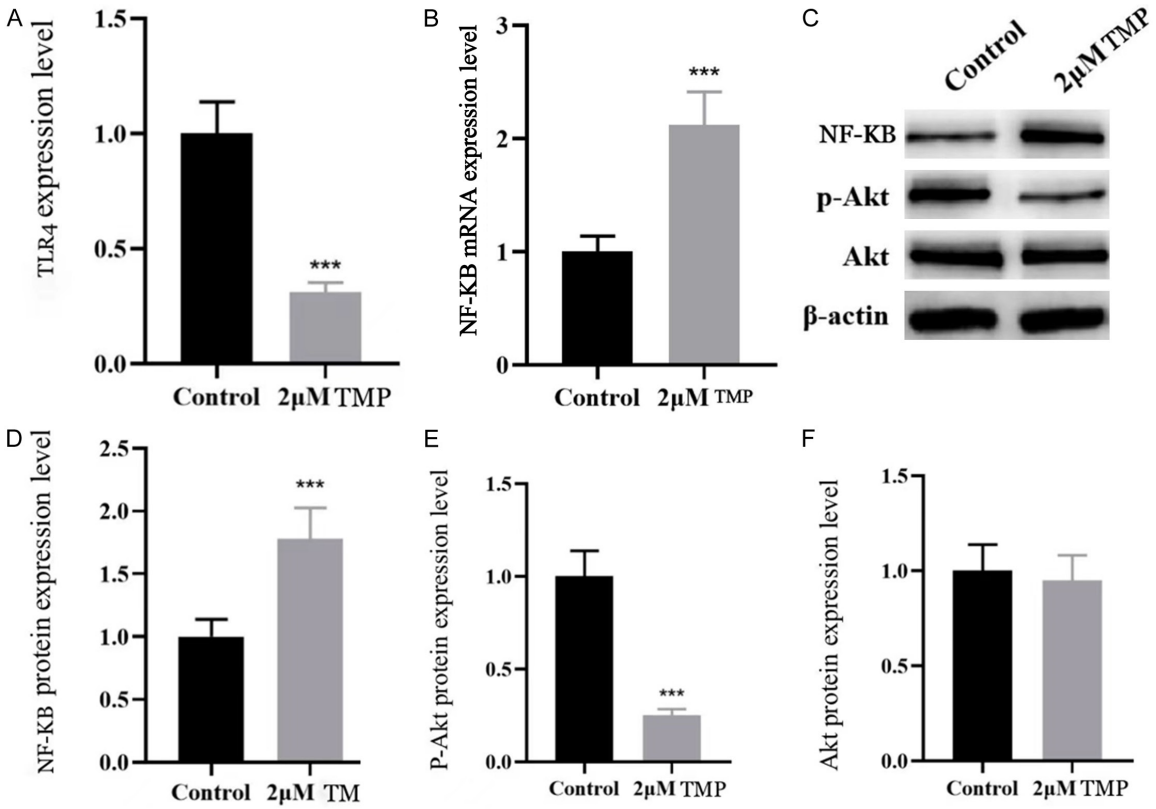

Figure 3. The effect of Ligustrazine on the expression of TLR4/NF-κB pathway-related molecules in cells. Note: (A and B) RT-PCR to detect the levels of TLR4 and NF-κB mRNA in cells treated with two μM of Ligustrazine for 48 h; (C-F) Western blot was used to detect the protein expression of NF-κB, p-Akt, and Akt in cells treated with 2 μM of Ligustrazine for 48 h. Compared to the control, ***P<0.001.

expression or localization of the target protein. According to the data obtained by fluorescence images or flow cytometry, quantitative analysis or image processing was performed to compare the difference between the different treatment groups.

Statistical analysis

SPSS25.0 software was used for statistical analysis, and the experimental results were expressed as mean ± standard deviation (X±s). One-way ANOVA followed with LSD test was used for data comparison among multiple groups. P<0.05 was considered significant.

Results

Ligustrazine inhibited the proliferation of STR cells

Ligustrazine demonstrated an IC_{50} of 0.5 μM in inhibiting cell proliferation. With an increase in Ligustrazine concentration, cell viability and clone formation were significantly decreased (all P<0.05) (**Figure 1**).

Ligustrazine promoted the apoptosis of STR cells

Flow cytometry showed that compared with the control group (0 μm), ligustrazine at a concentration of 0.5 μm and above significantly increased the apoptosis rate of STR cells (all P<0.001, **Figure 2A**, **2B**). Western blot showed that compared to the control group, treatment with 2 μM ligustrazine (2 μm TMP) significantly increased the protein expressions of Bax and cleaved Caspase 3 while it decreased the protein expression of Bcl-2 (all P<0.001, **Figure 2C-F**).

Effects of ligustrazine on the expression of TLR4/NF-κB pathway-related molecules in STR cells

RT-PCR showed that compared to the control group, 2 μM ligustrazine significantly decreased the mRNA level of TLR4 but increased the mRNA level of NF-κB in STR cells (all P<0.001, **Figure 3A**, **3B**). Western blot (**Figure 3C-F**) further demonstrated that compared with the control group, 2 μM ligustrazine significantly

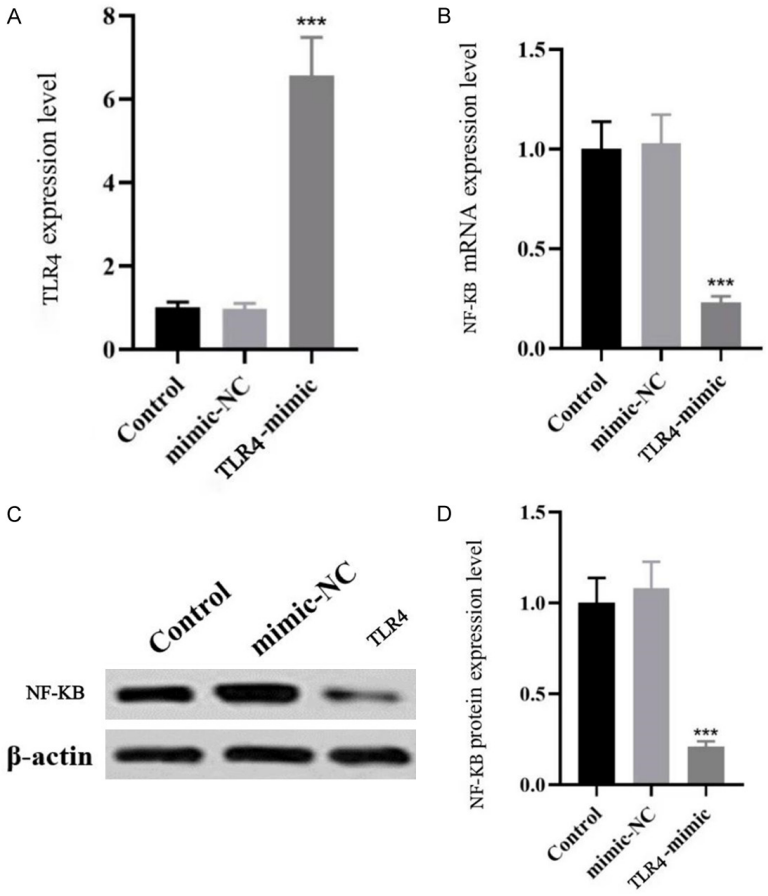

Figure 4. The regulatory effect of TLR4 on NF-κB. Note: (A and B) RT-PCR detection of TLR4 and NF-κB levels in cells transfected with TLR4-mimic or mimic-NC; (C and D) Western blot was used to detect the expression of NF-κB in cells transfected with TLR4-mimic or mimic NC. Compared to the control, ***P<0.001.

increased the protein expression of NF-κB but decreased the protein expression of p-Akt in cells (all P<0.001). However, 2 μM ligustrazine treatments did not remarkably change the protein expression of Akt (P>0.05).

TLR4-mediated regulation of NF-κB

Figure 4A illustrates that the TLR4 level in the cells transfected with TLR4-mimic (TLR4-mimic group) was significantly increased compared to the control group (P<0.001). In addition, compared to the control group, the mRNA and protein expression levels of NF-κB in cells transfected with TLR4-mimic were significantly decreased (**Figure 4B-D**).

TLR4/NF-κB pathway mediated the inhibitory effect of ligustrazine on cell proliferation

MTT assay and colony formation assay showed that compared to the control group, 2 μm TMP treatment significantly inhibited cell proliferation; Conversely, the proliferation ability of cells transfected with 2 μm TMP+TLR4-mimic was significantly increased (P<0.001, **Figure 5A-C**). In addition, flow cytometry analysis showed that 2 μm TMP treatment notably promoted apoptosis of cells compared to the control group; However, the apoptosis rate of cells transfected with 2 μm TMP+TLR4-mimic was decreased (P<0.001, **Figure 5D**, **5E**).

Cell immunofluorescence assay

Luciferase activity was employed to confirm that TLR4 could target NF-κB due to the specific binding site between NF-κB'-UTR and MiRNAs. The results showed that TLR4 mimics significantly inhibited NF-κB luciferase activity (P<0.05); However, there was no significant change in NF-κB luciferase activity without TLR4 mimic transfection (P>0.05), as shown in **Figure 6**.

Effects of ligustrazine on pain sensitivity in rats

Following surgery, the mechanical pain threshold of the model group was significantly decreased (P<0.01) (**Figure 7A**), and the cold pain sensitivity score was significantly increased (**Figure 7B**). At the same time, there was no significant difference in the two indexes before and after operation in the control group, indicating that the model rat model was successfully replicated.

Effects of intravenous ligustrazine injection on pain sensitivity in model rats

In this batch of experiments, the difference between different medication methods was not compared, so only the difference between the model group and the medication group was compared, only involving three groups.

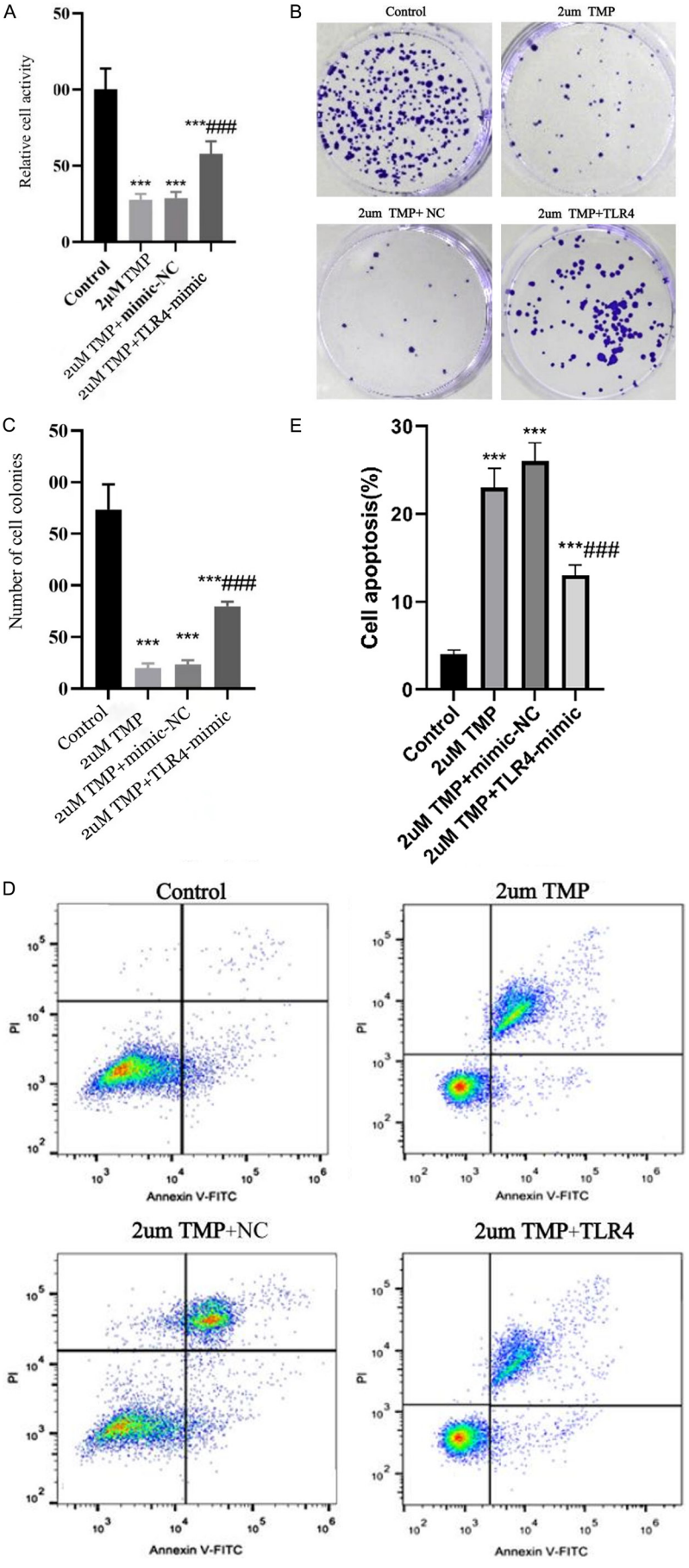

Figure 5. TLR4/NF-κB pathway mediates the inhibitory effect of Ligustrazine on cell proliferation. Note: (A) MTT method to detect the effects of 2 μM Ligustrazine and transfection with TLR4-mimic on the viability of cells; (B and C) Colony formation experiments to detect 2 μM Ligustrazine and transfection with TLR4-mimic effects on the proliferation of cells; (D and E) Flow cytometry to detect the effects of 2 μM Ligustrazine and TLR4-mimic transfection on cell apoptosis. Compared to control, ***P<0.001, compared to 2 μM TMP (Ligustrazine), ###P<0.001.

Compared with the model group, intravenous injection of ligustrazine (20 mg/kg) markedly improved the analgesic activity in model rats 60 min after injection (P<0.05) (**Figure 8A**). In contrast, the cold pain sensitivity score of model rats was significantly decreased at 60-120 min after injection (P<0.05) (**Figure 8B**).

Effects of ligustrazine on the content of amino acid transmitter in extracellular fluid

As shown in **Figure 9**, compared to the control group, the levels of Glutamate (Glu), Aspartate (Asp), and Glycine (Gly) in the extracellular fluid of were significantly elevated in the model group (all P<0.01). Similarly, the levels of Glu, Asp, and Gly in the extracellular fluid of medial prefrontal cortex (mPFC, A1-D1) and central amygdala (CeA, A2-D2) in model rats were higher than those in the control group. This increase was statistically significant before and 60-100 minutes after administration (P<0.05). However, there were no signifi-

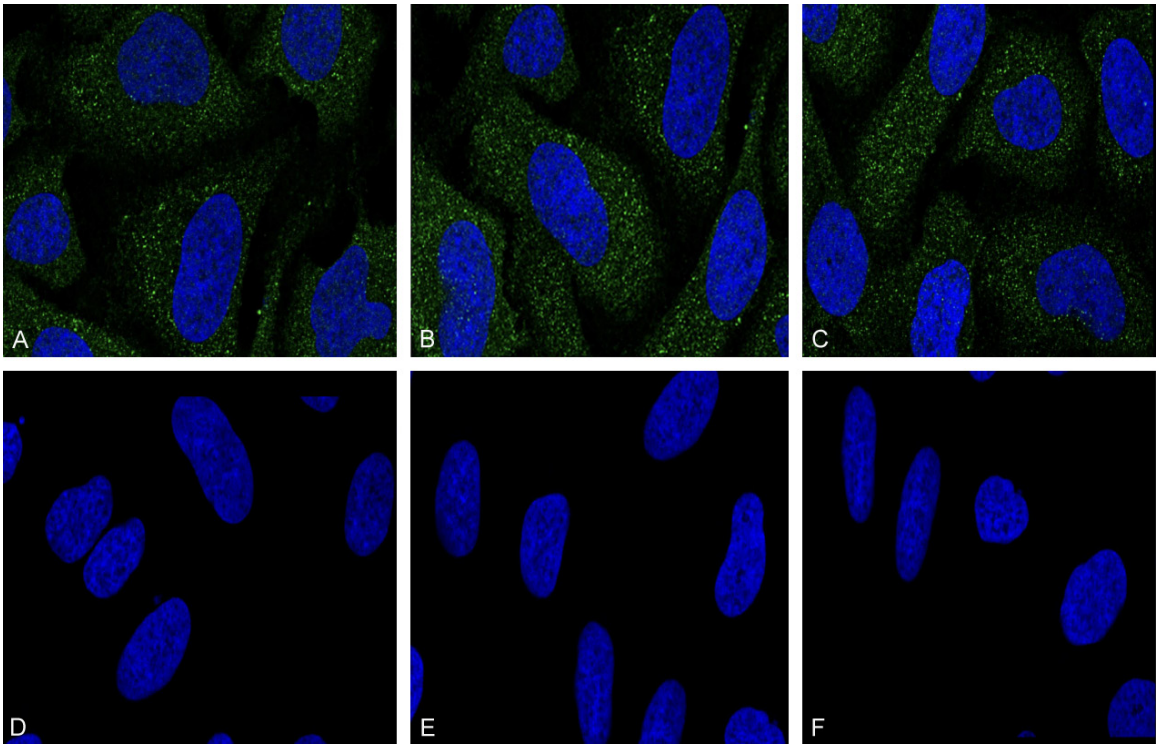

Figure 6. Cell immunofluorescence assay. A-C: Firefly luciferase signal: This is the main reporter gene that reflects the activity of the promoter region or regulatory element of interest. The intensity of the firefly luciferase signal was positively correlated with the transcript level of the NF-κB. Higher firefly enzyme signals indicate enhanced transcription; A: Control; B: Mimic-NC; C: TLR_4-mimic. D-F: VH renilla luciferase signal: This was an internal control gene that was used to correct for variability and transfection efficiency between samples.

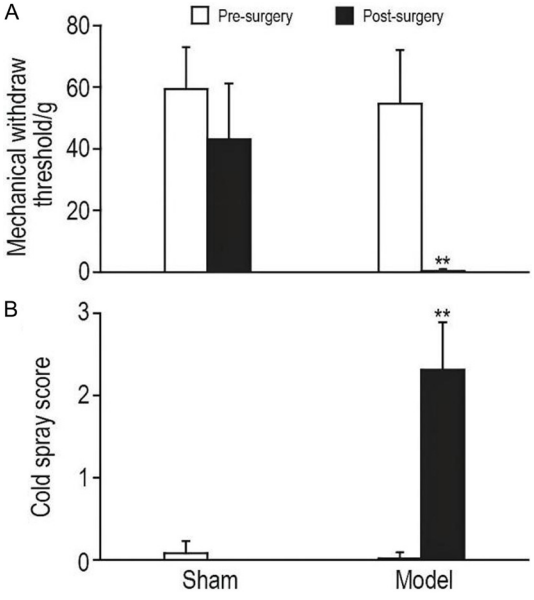

Figure 7. Evaluation of mechanical withdrawal threshold. Note: (A) Cold spray scores; (B) Before and after spared sciatic nerve injury in rats.

cant differences in Gamma-Aminobutyric Acid (GABA) content across all groups.

Discussion

Central sensitization refers to an abnormal increase in excitability or synaptic transmission of pain-sensing neurons in the spinal cord [19]. When peripheral nerves are injured, pain signals are transmitted to the dorsal horn of the spinal cord through A and C fibers. In the synapses of the dorsal horn, a large amount of glutamate (Glu), the primary excitatory neurotransmitter in the central nervous system (CNS), is released [20]. When Glu uptake is inhibited, it accumulates rapidly in the extracellular and synaptic spaces, intensively activating specific binding receptors, namely N-methyl-D-aspartic acid receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-isopropanoic acid receptor (AMPAR), on the postsynaptic membrane. This triggers a large influx of calcium ions (Ca^{2+}). Persistently high extracellular Glu levels result in an overload of intracellular Ca^{2+} ions, potentially causing neuronal cell death. Long-term and continuous excitation of the spinal cord and its higher centers (such as the cortex and thalamus) can induce changes in the CNS, a process known

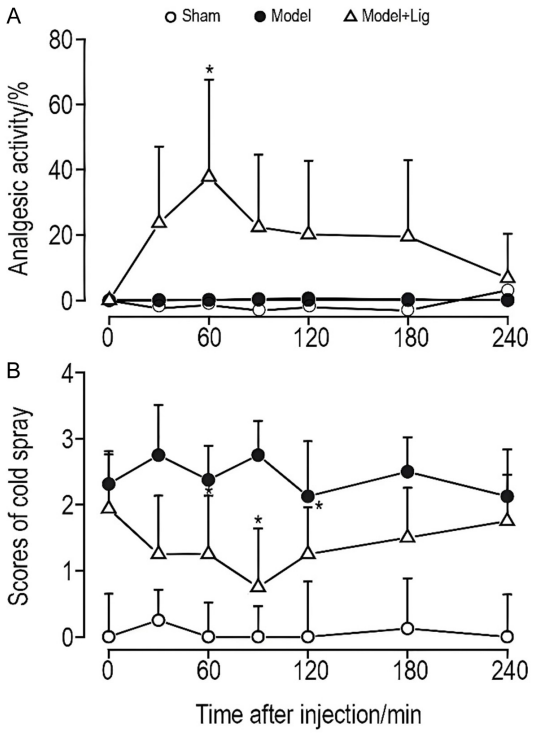

Figure 8. Analgesic effects of ligustrazine (20 mg·kg^{-1}). Note: Intravenous injection in model rats by mechanical withdraw threshold test (A) and cold spray test (B).

as NMDAR-mediated excitotoxicity, leading to central sensitization [21-24].

The postsynaptic activity of Glu neurotransmission triggered by NMDAR requires three simultaneous conditions [25]: (1) glutamate release from the presynaptic neuron into the synaptic cleft; (2) NMDAR activation through glutamate binding and co-agonists like glycine or D-serine; and (3) sufficient postsynaptic depolarization to remove the Mg^{2+} block on the NMDAR channel, allowing Ca^{2+} influx [26-28].

The amygdala and TLR4 are critical regions of pain signal conduction and experience in the spinal cord [29]. In this study, changes in excitatory and inhibitory amino acid neurotransmitters in the extracellular fluid of two brain regions (mPFC and CeA) of NPP model rats were dynamically observed by animal behavior and intracranial two-site microdialysis combined with high-performance liquid-fluorescence chromatography. The results showed that the mechanical pain threshold of the model group decreased significantly compared with the sham operation group, while the cold pain sensitivity score increased considerably. The contents of Glu and Gly in the extracellular fluid of model group increased significantly. However, the GABA/Glu ratio in the brain extracellular fluid of model rats tended to decrease. Pain-related behaviors of SSNI rats changed significantly before and after modeling, and the levels of Glu and Gly in the sensitized central brain regions were also significantly altered, suggesting that the SSNI rat model could be used as an ideal animal model to study neuralgia.

Additionally, regardingthe physiological function and pathological basis of the Glu system, Asp also increased synchronously with the increase of Glu. At the same time, the inhibitory neurotransmitter Gly increased obviously. However, there was no significant difference in GABA content. This may be the body's stress regulation response to maintain the dynamic balance between excitatory and inhibitory transmitters [30]. Furthermore, our research highlighted the potential therapeutic benefits of ligustrazine, a key component of Ligustrum Chuanxiong, in treating neuropathic pain. It is good at dispersing depression and passing blood and can warm away wind chill and remove wet turbidity. It can treat the symptoms of limb numbness and hemiplegia and also the symptoms of limb joint pain and adverse activity, so it is suitable for treating peripheral nerve damage [31-33]. Ligustrazine was shown to significantly reduce pain sensitivity and inhibit the up-regulation of P2X3 receptor expression, pointing to its potential as an effective treatment for neuropathic pain caused by spinal cord injury. Our results showed that intravenous administration of ligustrazine could significantly reduce the mechanical pain threshold and improve experimental rats' cold pain sensitivity. Moreover, intravenous administration of ligustrazine could dramatically inhibit the levels of Glu, Asp, and Gly in the extracellular fluid of mPFC and reduce the levels of Glu and Gly in the extracellular fluid of CeA. This suggests that the analgesic mechanism of ligustrazine on neuropathy pain caused by spinal cord injury may be related to the excitatory and inhibitory neurotransmitter.

Various studies have demonstrated that Ligustrazine can inhibit apoptosis and oxidative stress [34-37]. However, the underlying mechanism of the analgesic effect of ligustrazine on

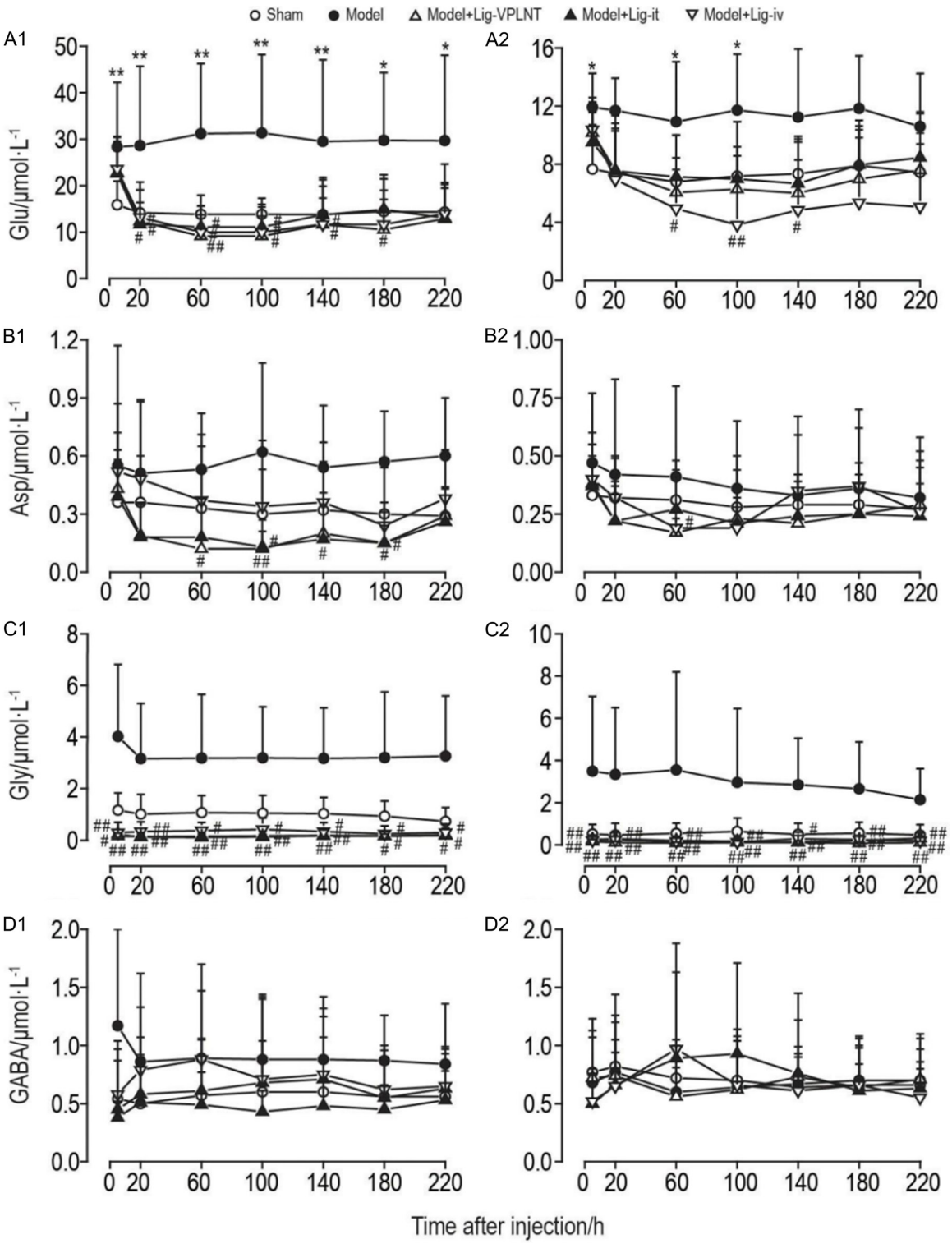

Figure 9. Effect of ligustrazine on extracellular contents of glutamate (Glu, A), aspartic acid (Asp, B), glycine (Gly, C) and γ-aminobutyric acid (GABA, D) in TLR4 and central nucleus of amygdaloid in model rats by high-performance liquid chromatography-fluorescence detector. Sham group: This was the control group, rats that underwent similar surgical procedures without actual nerve injury; Model group: This was the model group in the experiment, which underwent sciatic nerve injury surgery but received no other treatment or treatment; Model+Lig-VPLNT group: This group may have been treated with VPLNT after sciatic nerve injury; Model+Lig-it group: This group received CSF injection after sciatic nerve injury; Model+Lig-iv group: This group was treated with intravenous treatment after sciatic nerve injury.

neuropathic pain caused by spinal cord injury is unknown. It is hypothesized that ligustrazine may mitigate neuropathic pain caused by spinal cord injury by inhibiting the release of excitatory amino acid transmitters in the TLR4/NF-κB signaling pathway and by regulating the dynamic balance of excitatory and inhibitory amino acid transmitters. Therefore, an in-depth study of the therapeutic effect and mechanism of ligustrazine on NPP rats has crucial clinical significance and would provide a new scientific basis for repurposing this drug.

However, this study has some limitations: Only the STR cell line was used; therefore, the results may not be generalized to other cell lines or tissues. Experiments were only conducted in vitro and in animal models, lacking direct validation in humans. Only some cell and protein parameters were measured, potentially overlooking other factors related to pain sensitivity. Additionally, possible experimental errors and random variations were noted, indicating a need for further research to ensure the stability and reproducibility of these findings. Detailed information about the control group was not mentioned, making it difficult to determine whether the observed effects were caused by ligustrazine treatment or other factors.

Conclusion

The analgesic effect of Ligustrazine on neuropathic pain caused by spinal cord injury may be related to its inhibition of the release of excitatory amino acid transmitters Glu and Gly in the TLR4/NF-κB pathway, regulation of the dynamic balance of excitatory and inhibitory amino acid neurotransmitters, and alleviation of the central sensitization effect caused by the excitotoxicity of Glu.

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Disclosure of conflict of interest

None.

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