

Melatonin Attenuates Pyroptosis Upon Spinal Nerve Ligation in Rats via the NF-κB/NLRP3 Inflammasome Signaling Pathway

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Research

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Abstract

Accumulated evidences have demonstrated causative links between neuropathic pain (NP) and immune-mediated inflammatory disorders. However, the role of inflammasome-induced pyroptosis in NP remains elusive. Melatonin possesses a well-documented analgesic action in various pain models. A rat model of spinal nerve ligation was established to explore the potential mechanism of melatonin in pyroptosis. The current study aimed to test our hypothesis that melatonin regulated pyroptosis to alleviate NP by inhibiting NF- κ B/NLRP3-dependent signaling. Behavioral experiments revealed that SNL provoked severe allodynia which were suppressed by the administration of melatonin, caspase-1 inhibitor (VX-765) or NF- κ B inhibitor (BAY 11-7085). SNL significantly up-regulated the inflammatory cytokines associated with the excessive activation of NLRP3 components and NF- κ B signaling, as well as the marked pyroptosis activation which were partially inhibited by melatonin, VX-765 or BAY 11-7085. Collectively, Melatonin has potent analgesic and anti-inflammatory effects in SNL models through preventing pyroptosis via the NF- κ B/NLRP3 inflammasome signaling pathway.

Introduction

Neuropathic pain (NP), characterized by difficult treatment decisions and poor outcomes, has become a public health epidemic all over the world, with its long-term, chronic discomfort and pain seriously affecting patients' quality of life (Gierthmühlen & Baron, 2016). To date, the accumulated evidence has demonstrated the critical role of neuroinflammation in the pathogenesis of NP which leads to enhanced pain sensitivity and is characterized by hyperalgesia and allodynia (Jiang et al., 2016; Huang et al., 2018). In view of the complexity of neuropathic symptoms and the resistance to treatment of NP, these published studies provide crucial insights for this field.

The nucleotide-binding oligomerization domain-like receptors family pyrin domain containing 3 (NLRP3) inflammasome is a multimeric protein complex, which includes the adaptor apoptosis-associated speck-like protein containing a CARD (ASC) and the effector pro-caspase-1 (Elliott & Sutterwala, 2015). When cells are exposed to danger signals, the NLRP3 inflammasome drives caspase-1 activation, which leads to the upregulation of mature IL-1 β and an extended immune response (Wang et al., 2020; Dong et al., 2019). Although several studies (Wang et al., 2020; Pan et al., 2018) have demonstrated that NLRP3 participates in the on-set and continuation of NP, the molecular biological frame mechanisms regarding upstream and downstream effectors of NP remain to be elucidated.

Pyroptosis is emerging as a unique inflammatory-regulated cell death mechanism that is dependent on caspase-1 activity and has been reported to be involved in various chronic inflammatory disease (Zhang et al., 2018; McKenzie et al., 2018). Gasdermin D (GSDMD) triggers pyroptosome formation and caspase-1-mediated pyroptosis, forming membrane pores to induce the release of proinflammatory cytokines (Sborgi et al., 2016; Xu et al., 2018). However, the underlying mechanisms by which caspase-1-mediated pyroptosis participates in spinal nerve ligation (SNL)-induced NP remain unclear.

Nuclear factor-kappa B (NF- κ B) is a transcription factor that takes a pivotal part in the onset of inflammation (Mitchell & Carmody, 2018). The NLRP3 inflammasome increases the production of inflammatory mediators by activating NF- κ B signaling during chronic inflammation (Fann et al., 2018). Recent studies have reported that GSDMD-related pyroptosis mediated by TLR4/NF- κ B signaling may be associated with the onset and progression of chronic inflammatory diseases (Wang et al., 2019). Therefore, in the present study, we hypothesized that cell pyroptosis is induced in an NLRP3-dependent manner during the progression of SNL which is mediated by the NF- κ B signaling pathway.

Melatonin (N-acetyl-5-methoxytryptamine), an endogenous and synthetic hormone, plays a protective role in various inflammatory disorders owing to its powerful anti-inflammatory properties (Hardeland, 2019; Ping et al., 2017). It has been demonstrated that melatonin alleviated inflammasome-triggered pyroptosis through the GSDMD-dependent NF- κ B signaling pathway in adipose cells (Liu et al., 2017). Moreover, Galley et al. have shown that oral administration of melatonin alleviates pain behaviors in a rat model of paclitaxel-induced peripheral neuropathy (Galley et al., 2017). These studies implied that melatonin might play a prominent role in the development of NP by regulating cell pyroptosis, which is mediated by the NF- κ B signaling pathway. Thus, this is the first time that such a concept has been experimentally proposed.

In this study, we explored the effects of melatonin on cell pyroptosis in a rat model of SNL-induced NP. We also aimed to get insight into how the potential NF- κ B/NLRP3 inflammasome signaling mechanisms are affected upon treatment of NP with melatonin.

Methods And Materials

Experimental Animals and Experimental Groups

Sprague-Dawley (SD) rats (male, weighing 200-250g) used in this study were supplied by Shandong University Experimental Animal Center (Shandong, China). The rats were reared in groups of about 3-5 rats. Each group was subjected to a 12-hour light/dark cycle and bred with standard rodent chow and water. The ambient temperature was controlled at ($25\pm1^{\circ}\text{C}$) and humidity at ($45\pm5\%$), respectively. The entire animal trial was approved by the Qingdao University Animal Care and Use Committee.

The intraperitoneal injection was performed with melatonin (20mg/kg, MedChemExpress, HY-13205), vehicle (i.e. an equivalent volume of phosphate-buffered saline (PBS)), caspase-1 inhibitor VX-765 (50mg/kg, MedChemExpress, HY-B0075) or the inhibitor of NF- κ B activation BAY 11-7085 (BAY) (5mg/kg, MedChemExpress, HY-10257) within the first three consecutive postoperative days.

Spinal nerve ligation Model

The well-established L₅₋₆ SNL rat model of neuropathy was used in this study. The procedure was performed as follows: the experimental animals were placed on a sterile operating table in a prone

position and anesthetized by sodium pentobarbital (50 mg/kg, intraperitoneally). The skin of the back was incised longitudinally and the left paraspinal muscles were segregated from spinous processes. The left L₅₋₆ inter-laminar space was exposed, and the spinal nerves were tightly ligated with 6-0 silk sutures after carefully separating them from the surrounding tissues. The sham group was subjected to all surgical procedures, except the nerve ligation. All rats were placed post-surgery in a warming chamber with individual cages and body temperatures were maintained at approximately 38°C until they were completely awake. Then, on the 7th postoperative day rats from different groups were sacrificed and the spinal cord tissues from the ipsilateral lumbar enlargement were dissected and cryo-preserved at -80°C. All surgical procedures were performed aseptically and carried out in the morning to avoid diurnal variations on the immune response.

Assessment of mechanical hyperalgesia

All rats were assessed for mechanical hyperalgesia one day before surgery and on 7 consecutive days post-surgery. Before the assessment, all experimental rats were individually kept for an hour in a quiet room to acclimatize to the environment. We used the 50% paw withdrawal latency (PWL) to assess mechanical sensitivity as described previously (Chaplan et al., 1994). Von Frey filaments (Stoelting, USA) were utilized to stimulate the fourth or fifth metatarsal surface of the right posterior hind paw following the “up-down” calculating approach. The stimulus was applied for 6-8 seconds and this was repeated five times in 10-15-min intervals to avoid sensitization. A positive response included the rapid withdrawal or lapping of the paw and the lowest stimulus that evoked a clear positive reaction was accepted as the threshold. Testing was performed by a single experienced investigator, who was blinded to treatment the rats received, at approximately the same time of the day.

Enzyme-Linked Immunosorbent Assay (ELISA).

Protein levels of inflammatory factors in the ipsilateral spinal cord horns were assessed by ELISA. The spinal cord samples were homogenized in 150 µl of RIPA lysis buffer and centrifuged at 20,000 r/min for 30 min at 4°C. Interleukin (IL)-1β and IL-18 secretion in each group was determined from the supernatant using ELISA kits (RD Systems, USA; ExCell, China) following the manufacturer's instructions. Absorbance measurements were read at 450 nm wavelength using a microtiter plate reader. We used a standard calibration curve from standards provided by the manufacturer to calculate the antigenic values for the cytokines.

Western blot analysis

Total proteins from samples were extracted in RIPA lysis buffer (Beyotime Biotechnology, China). Nuclear proteins were extracted using a nuclear protein extraction kit (Thermo Scientific). Following incubation for 1h and centrifugation at 12,000 g (4°C, 20min), the supernatant was collected. Then, protein samples

were separated using 8-12% SDS-polyacrylamide gels (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Boston, MA, USA). After blocking in 5% non-fat milk for 1h (25°C), membranes were incubated with the following specific primary antibodies at 4°C overnight: anti-NF-κB p65 antibody (Abcam, ab86299), NLRP3 antibody (Proteintech, 19771-1-AP), ASC/TMS1 polyclonal antibody (ABclonal, A16672), caspase-1/P10 antibody (Proteintech, 22915-1-AP). After washing with TBS-T buffer, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit or anti-mouse, 1:2000; Beyotime Biotechnology) at room temperature (25°C) for 1h. The immunolabeled proteins were detected using the Enhanced Chemiluminescence Plus kit (Millipore, USA) with LAS-4000 mini (Fuji, Japan). The level of β-actin was used as a loading control.

Analysis of cell death

Pyroptosis was assessed using propidium iodide (PI) fluorescent staining. The spinal cord tissues were collected and cryo-preserved at -80°C on the 7th-day post-SNL. The PI dye (1 mg/kg, 100ul) was perfused intraperitoneally one hour before the rats were killed. The frozen spinal cord tissues (7μm) were cut into sections on a freezing microtome. Sections were blocked with 5% fetal bovine serum for 1h in PBS containing 0.1% Triton X-100 (Sigma) in the dark at room temperature. Subsequently, the sections were incubated in the presence of the primary antibody (anti-caspase-1, 1:100) at 4°C overnight. Following flushing with PBS (three times), the sections were incubated with the proper secondary antibody (Dylight 488, 1:200, Abcam, USA) in the dark for 2 h. The nuclei were counterstained with DAPI (1:1000, Sigma, USA) for 5 min. The stained sections were imaged under a fluorescence microscope (Olympus, Japan).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total mRNA was extracted from spinal cord tissues with TRIzol Reagent (Life Technologies, USA). We used a Prime Script RT reagent kit (Takara, Dalian, China) to performed PCR and the SYBR-Green Premix Ex Taq (TaKaRa, Dalian, China) on a Light Cycler® 480 Instrument II (Roche, Switzerland) to detect the relative mRNA levels. The expression of the target gene sequences was normalized to β-actin. The data were quantified using the $2^{-\Delta\Delta}$ method. Table 1 shows the sequences of gene-specific primers used in this study.

Table 1
The Primer Sequences for RT-PCR

Gene	Length	Primer	Sequences
NLRP3	291bp	Forward	5' CTGCATGCCGTATCTGGTTG 3'
		Reverse	5' CGCGTGTAGCAGAAATCCAG 3'
ASC	188bp	Forward	5' CCATCCTGGACGCTCTGAAA 3'
		Reverse	5' TGTGAGCTCCAAGCCATACC 3'
Caspase 1	137bp	Forward	5' GAACAAAGAAGGTGGCGCAT 3'
		Reverse	5' CAAGACGTGTACGAGTGGGT 3'
β -actin	138bp	Forward	5' TTACTGCCCTGGCTCCTAG 3'
		Reverse	5'CGTACTCCTGCTTGCTGATC3'

Statistical analysis

All data were presented as mean \pm standard deviation (SD). SPSS statistical software system (version 20.0 for Windows, Chicago, IL) was used for data analysis. Comparisons of the levels of pyroptosis, NF- κ B signaling, expression levels of the NLRP3 inflammasome components, and inflammatory cytokines in response to melatonin, VX-76, and BAY treatments were carried out using analysis of variance (ANOVA). $P < 0.05$ was set as the threshold for statistical significance.

Results

Melatonin-promoted pain relief and reduced inflammatory response

The effect of SNL on the mechanical hypersensitive thresholds was examined. The withdrawal thresholds had significantly decreased compared with the pre-surgery baseline and the sham group (Fig. 1 A). We then assessed the mechanical withdrawal thresholds after melatonin administration. Compared with the vehicle group, melatonin injection significantly elevated the mechanical withdrawal threshold in the SNL treatment group (Fig. 1 A). However, there was no difference between the sham groups with or without melatonin (Fig. 1 A).

Then, to investigate whether the decreased withdrawal threshold following SNL was due to inflammation and to assess the effect of melatonin, we determined the expression levels of IL-18 and IL-1 β by ELISA. As shown in Fig. 1B and C, the production of both pro-inflammatory factors was significantly elevated in the spinal cord following SNL compared to the sham group. Meanwhile, melatonin administration was effective in suppressing the increased production of IL-18 and IL-1 β compared with the vehicle group (Fig.

1B and C). Moreover, the levels of pro-inflammatory cytokines in the sham group remained unchanged following melatonin administration.

Melatonin attenuates SNL-induced NLRP3 inflammasome activation

Since an important function of the NLRP3 inflammasome activation is to promote the maturation of pro-inflammatory cytokines, we tested whether melatonin prevented the activation of NLRP3 inflammasome-related molecules in SNL-induced NP models. Analysis by RT-PCR showed that the mRNA expression levels of NLRP3, ASC, and caspase-1 P10 were significantly enhanced in the vehicle group compared with the sham group but were inhibited by melatonin administration as shown in Fig. 2A. Meanwhile, similar results were uncovered using a Western blot assay demonstrating that melatonin administration alleviated the up-regulation of caspase-1 P10, NLRP3, and ASC induced by SNL (Fig. 2B). However, we did not observe any statistically significant difference between the sham groups with or without melatonin. Moreover, the expression of caspase-1 P45 was unchanged (Fig. 2B).

Melatonin mitigates SNL-induced GSDMD activation and pyroptosis

As shown in Fig. 3A, Western blotting analysis demonstrated that the protein expression of GSDMD was markedly increased upon SNL but was alleviated after melatonin treatment. However, there was no statistically significant difference between the sham groups with or without melatonin treatment.

To further confirm the presence of pyroptosis, we performed immunohistochemistry. The presence of cleaved-caspase-1 and PI-positive cells indicated pyroptosis. Our immunohistochemistry results showed that SNL induced a high cell death rate along with the elevation of cleaved caspase-1, which was reversed upon melatonin treatment (Fig 3B). Therefore, our results demonstrated that melatonin could regulate the GSDMD-mediated pyroptosis.

Caspase-1 inhibitor blunted spinal cord pyroptosis and alleviated SNL-induced mechanical hyperalgesia and inflammatory response

We next investigated whether injection of the specific caspase-1 inhibitor VX-765 could also mitigate the key inflammasome and pyroptosis activation in an SNL-treated rat model. Our Western blotting results suggested that, similar to melatonin, VX-765 effectively blocked the induction of high levels of expression of caspase-1 P10, NLRP3, and ASC, and of GSDMD upon SNL treatment (Fig 4A).

We further examined the role of VX-765 in regulating inflammasome-triggered pyroptosis using immunohistochemistry staining. The results we obtained were consistent with those of the Western blotting assay. Administration of VX-765 led to down-regulation of the SNL-induced positive expression of caspase-1 and pyroptosis (Fig 4B). In addition, we found that melatonin displayed a more potent inhibition relative to VX-765.

We further tested the inflammatory reaction upon intraperitoneal injection of VX-765 using ELISA. We found that the expression of the pro-inflammatory cytokines increased markedly following SNL, while administration of either VX-765 or melatonin inhibited the SNL-induced secretion of IL-18 and IL-1 β .

Next, we investigated the effect of caspase-1 activation on mechanical pain allodynia and whether VX-765 had analgesic effects similar to melatonin on mechanical hypersensitivity. Consistent with the effects of injection of melatonin, administration of VX-765 reversed the lowering of the mechanical withdrawal threshold that was seen upon SNL-induced inflammation.

Taken together, suppressing the NLRP3-mediated pyroptosis could promote inflammatory reaction resolution and control pain. The addition of melatonin improved radicular pain by inhibiting the activation of pyroptosis.

Melatonin reduces SNL-induced mechanical algesesthesia mediated by the NF- κ B/NLRP3 signal pathway

Taking into consideration the critical role of the NF- κ B pathway in NP, we used Western blotting to explore whether NF- κ B signaling is over-activated in our SNL-treatment rat model of NP. As seen in Fig. 6A, the phosphorylation of NF- κ B was markedly elevated in the spinal cord of rats upon SNL compared with sham-treated rats, whereas melatonin administration suppressed the increase of NF- κ B phosphorylation upon SNL.

Similar to the administration of melatonin, we found that BAY treatment dramatically inhibited the components of the NLRP3 inflammasome and its downstream effector GSDMD in the spinal cord of the rat SNL model compared with the control group (Fig. 6B). Similarly, the mRNA levels of caspase-1 P10, NLRP3, ASC, and GSDMD were also markedly elevated upon SNL but were significantly reduced following BAY injection compared to the vehicle group (Fig. 6B). However, we did not find any statistically significant difference between the melatonin treatment group and the melatonin and BAY co-treatment group.

Finally, we assessed the effect of treatment with BAY on SNL-induced mechanical hyperalgesia. In the BAY or melatonin-administrated group, mechanical hyperalgesia was significantly recovered compared with the SNL-treated control group. Furthermore, co-treatment of BAY and melatonin marked alleviated the reduction of the mechanical withdrawal thresholds (Fig. 6C).

Discussion

Throughout the current study, we demonstrated that melatonin has potent anti-inflammatory and analgesic properties in a rat NP model. Additionally, we identified the critical contribution of the NLPR3 inflammasome to the development of SNL-induced NP, which was associated with the production of the pro-inflammatory cytokines. These results explained our previous findings that the inflammatory response triggered by the NLPR3 inflammasome critically participated in NP (Wang et al., 2020). Furthermore, we found that the close connection between NLRP3 inflammasome-mediated pyroptosis and NF- κ B signaling is an underlying mechanism for the analgesic effect of melatonin. Thus, the NF- κ B/NLRP3/pyroptosis signaling pathway is an attractive drug for the clinical application of melatonin for the prevention of NP.

NP is a complex chronic inflammatory disease, and there are no available therapeutic strategies to completely control the chronic pain. Although the inflammatory nature of NP has already been well studied, the molecular signaling mechanisms behind the inflammatory response leading to NP are still incompletely clarified. In keeping with previous studies, we have successfully established a rat SNL pain model which produced persistent pain hypersensitivity (Yang et al., 2019). Previous research has shown that NP is immunologically active and involved in the activation of various inflammasomes during the process of chronic inflammation (Tonkin et al., 2018). Consistent with these results, our present study demonstrated that the behavioral changes were correlated with elevated production of IL-18 and IL- β in the spinal cord.

The NLRP3 inflammasome is one of the indispensable multimeric protein complexes that regulate the autoimmune system, which then leads to the secretion of pro-inflammatory molecules, for example IL-1 β and IL-18 (Christ et al., 2018). Grace et al. (Grace et al., 2016) reported that the initiation and continuation of NP were mediated by morphine-induced NLRP3 inflammasome activation in the spinal cord and the associated release of IL-1 β in microglia. The relationship between inflammatory pain and the NLRP3 inflammasome has been well studied, including during chemotherapy and peripheral nerve injury-induced NP (Tonkin et al., 2018; Jia et al., 2017). Previous reports have demonstrated that melatonin is able to suppress the inflammatory process and improve NP (Tonkin et al., 2018; Lin et al., 2017; Hsieh et al., 2017). In the current study, we found that intraperitoneal injection of melatonin prevented the upregulation of the NLRP3 inflammasome and the subsequent activation of pro-inflammatory cytokines. Intriguingly, in parallel with the downregulation of IL-18 and IL-1 β upon administration of melatonin, our behavioral data also showed that melatonin treatment markedly reversed the paw withdrawal threshold in rats with SNL-induced NP. These data demonstrated SNL evokes an immune system response resulting in a persistent inflammatory cascade and sensitization of nociceptive receptors in models of NP, and that melatonin administration may be used as a practical treatment to regulate the immune reaction and ease the pain.

Recent work has shown that NLRP3 inflammasome activation engages a kind of inflammatory cell death, named pyroptosis, in immune cells (He et al., 2016), typically characterized by the release of pro-

inflammatory molecules (Shi et al., 2017). Gasdermin D (GSDMD), a reliable marker of pyroptosis, is involved in various inflammatory disease processes (Ding et al., 2016). However, the mechanism underlying NP-induced pyroptosis remained obscure. Our experiments demonstrated that SNL-induced NP increased caspase-1 P10, NLRP3, ASC, and GSDMD expression and resulted in pyroptosis in contrast to the sham group. This increase was suppressed by the administration of melatonin. Therefore, we speculate that melatonin alleviated the neuronal inflammatory response by suppressing the expression of pyroptosis. Taken together, these results imply that pyroptosis, an inflammatory form of regulated cell death, could present a target for melatonin-mediated inhibition of neuronal inflammation.

Caspase-1, executing the central role in the NLRP3 pathway, is responsible for the active process of inflammatory factors, which are pivotal mediators of innate immunity and critically take part in various inflammatory diseases (Van Opdenbosch & Lamkanfi, 2019). Therefore, we next performed an inhibitory experiment with a specific caspase-1 inhibitor (VX-765) to determine whether a similar mechanism applies to caspase-1-dependent pyroptosis activation that occurs during the progression of NLRP3 inflammasome-related NP. Here, we validated that NLRP3, ASC, and caspase-1 synthesis and the activation of pyroptosis were promoted in an SNL-induced NP model and that these effects were neutralized by the caspase-1 inhibitor, suggesting that SNL-induced pyroptosis was dependent on the activation of the NLRP3 inflammasome. Moreover, the mechanical sensitivity threshold had improved following the application of VX-765 similar to the effect of melatonin. Thus, our results showed that the NLRP3 inflammasome-activated pyroptosis is a key target for the alleviation of NP using melatonin.

Although it is well known that inflammatory caspase-1 is associated with pyroptosis, the mechanism of the NLRP3 inflammasome-induced pyroptosis in NP is largely unclear. The NF- κ B transcription factor is involved in the cellular signaling pathway in inflammation-related disease. Several reports have suggested that the NF- κ B pathway directly relates to the development of NP. Zhang et al (Zhang et al., 2013) showed that NF- κ B activation was ascended in rat lumbar DRG neurons following type 2 diabetes mellitus-induced NP. Zhong et al (Zhong et al., 2020) have shown that bone marrow mesenchymal stem cells down-regulate the NF- κ B pathway to alleviate deafferentation pain in rats. Recently, a revolutionary idea has been put forward suggesting that induction of pyroptosis is accompanied by NF- κ B activation during inflammation (Yao & Sun, 2019). Therefore, we examined the role of the NF- κ B signaling pathway in the inflammatory process in an SNL rat model. We found an increase in NF- κ B phosphorylation in the spinal cord in the rat model of SNL-induced NP and a down-regulation of NF- κ B expression upon melatonin treatment. BAY 11-7085 (Juliana et al., 2010), as a potent and specific NF- κ B signaling pathway inhibitor, has been shown to possess anti-inflammatory and neuroprotective activities. Interestingly, a key finding of the current study demonstrated that the increased biosynthesis of pyroptosis-related proteins, including NLRP3, ASC, caspase-1, and GSDMD were in each case reversed or blunted in the presence of BAY 11-7085 and also reversed pain-related behavior in the rat pain models of SNL, demonstrating that SNL activated the NF- κ B signaling pathway to accelerate one or more of the signaling molecules upstream of the NLRP3 inflammasome-mediated pyroptosis cascade in the SNL-induced NP model. Thus, these data revealed the possibility that melatonin alleviated NP, in part, due to

the suppression of inflammasome-mediated pyroptosis which may be regulated by the NF-κB signaling pathway.

In conclusion, the purpose of our study was to elucidate the analgesic effect of melatonin in the regulatory molecular signaling involved in the inflammatory reaction. Our results showed that the NF-κB pathway and NLRP3 inflammasome-mediated pyroptosis played key roles in the onset and perpetuation of SNL-induced NP. Furthermore, melatonin and an NF-κB inhibitor attenuated the NLRP3 inflammasome- and pyroptosis activation and promoted pain-relief in NP by inactivating the NF-κB signaling pathway. A mechanism targeting the NF-κB pathway and the NLRP3 inflammasome-mediated pyroptosis might be a prospective therapeutic avenue for the prevention and treatment of NP. However, this being one of the mechanisms towards the generation of NP, further studies are needed to uncover the specific pathogenesis processes.

Declarations

Ethics approval and consent to participate:

The animal experiments were approved by the Qingdao University Animal Care and Use Committee.

Consent for publication:

Not applicable.

Availability of data and material:

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

Competing interests:

All the authors declare no financial or commercial conflicts of interest.

Funding:

The study received no funds.

Authors' contributions:

Drs. Yi-hao Wang and Xiao Gao were responsible for carrying out the major part of the study and writing the manuscript. Dr. Yu-ru Tang carried out the statistical analyses and helped conduct the animal models and western blot study. Dr. Zhao-jun Liang helped carry out the behavioral tests. Drs. Nan-nan Zhang and Juan Liu helped carry out the ELISA study. Dr. Yan Li conceived and designed the study, and revised the manuscript.

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Figures

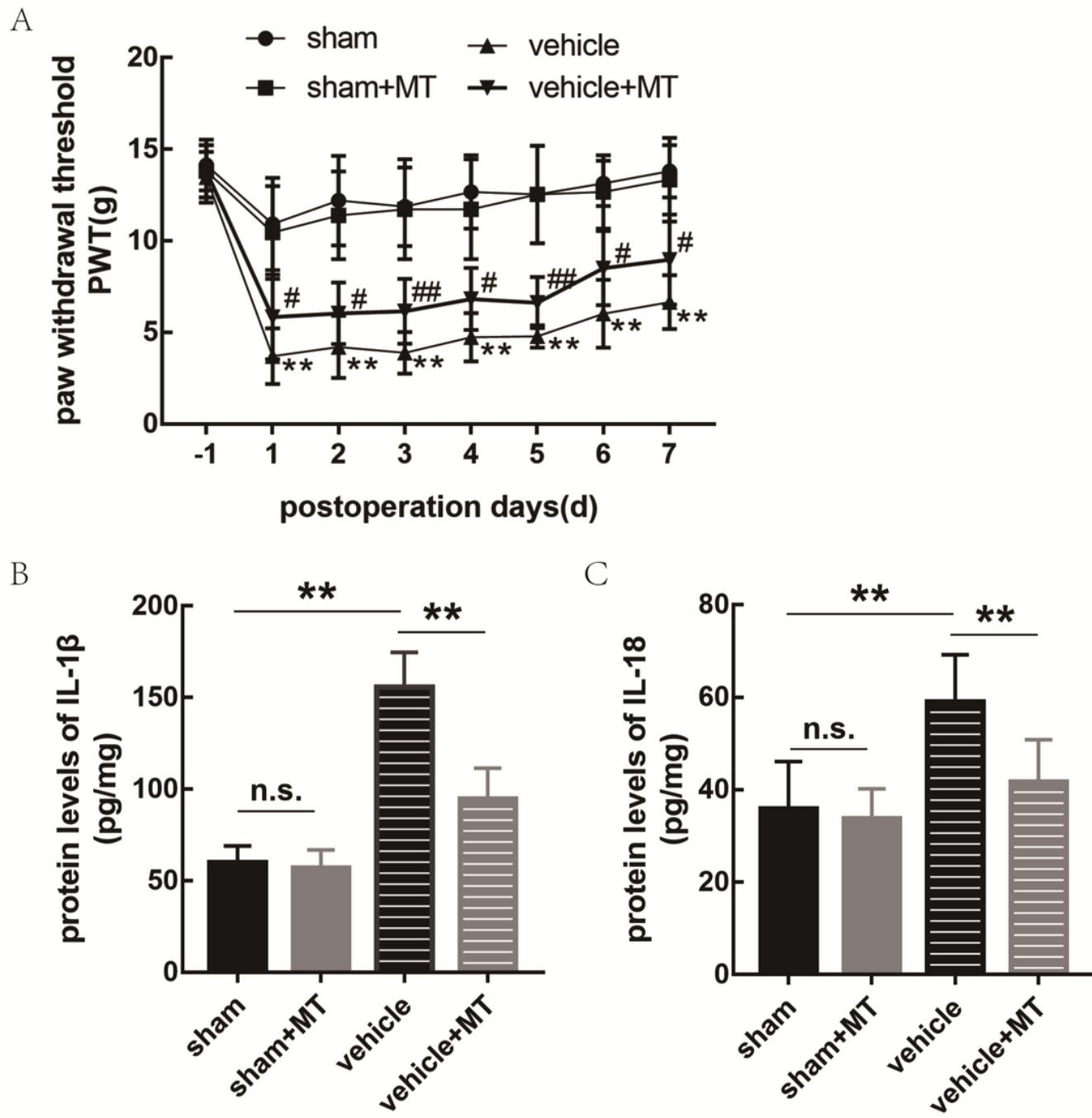


Figure 1

Effect of melatonin injection on the expression of pro-inflammatory cytokines in ipsilateral spinal cord horn and mechanical pain hypersensitivity induced by spinal nerve ligation. A Time course analysis of behavior demonstrating the effect of melatonin on SNL-induced hyperalgesia. The withdrawal threshold against von Frey stimulation was recorded one day prior to surgery and every day postoperatively until day 7 (n =8/group). Melatonin alleviated the reduction of PWT post-surgery. **P < 0.01 versus vehicle group; #P< 0.05 versus vehicle + MT group; ##P< 0.01 versus vehicle + MT group. B, C The expression levels of inflammatory factors in the ipsilateral spinal cord horns were examined by ELISA (n =6/group). Melatonin intraperitoneal injection reversed the increase of IL-1- β and IL-18 levels induced by SNL. **P < 0.01; n.s., no significant difference.

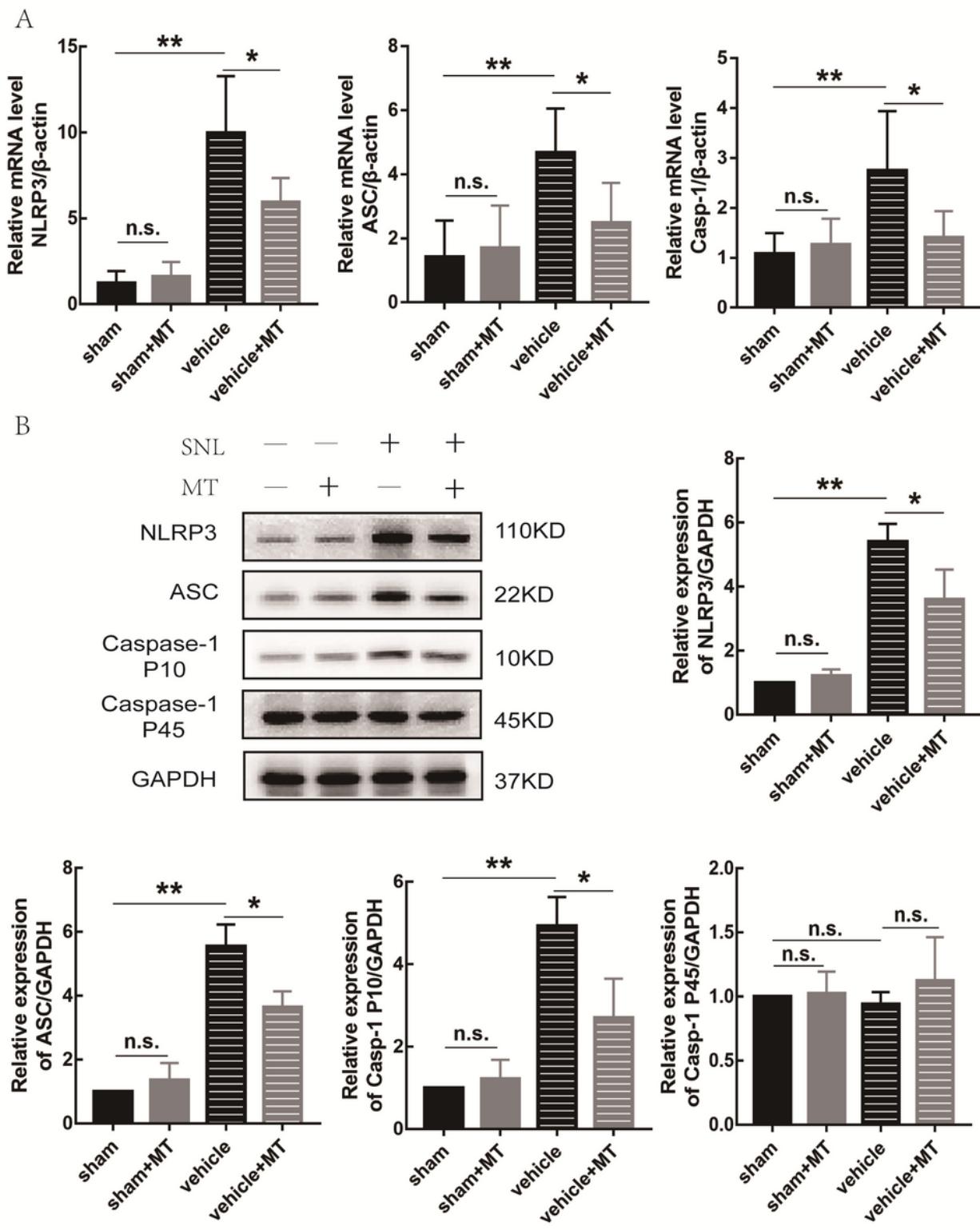


Figure 2

Melatonin reduced the enhanced expression of the NLRP3 inflammasome components in SNL-treated rats A RT-PCR analysis demonstrated that caspase-1, NLRP3, and ASC mRNA expression levels had markedly increased following SNL, which was reversed upon melatonin injection ($n = 6/\text{group}$). B Western blot results indicated that the levels of caspase-1, NLRP3, and ASC were significantly higher following

SNL operation which was prevented by melatonin administration ($n = 6$ /group). ** $P < 0.01$; * $P < 0.05$; n.s., no significant difference.

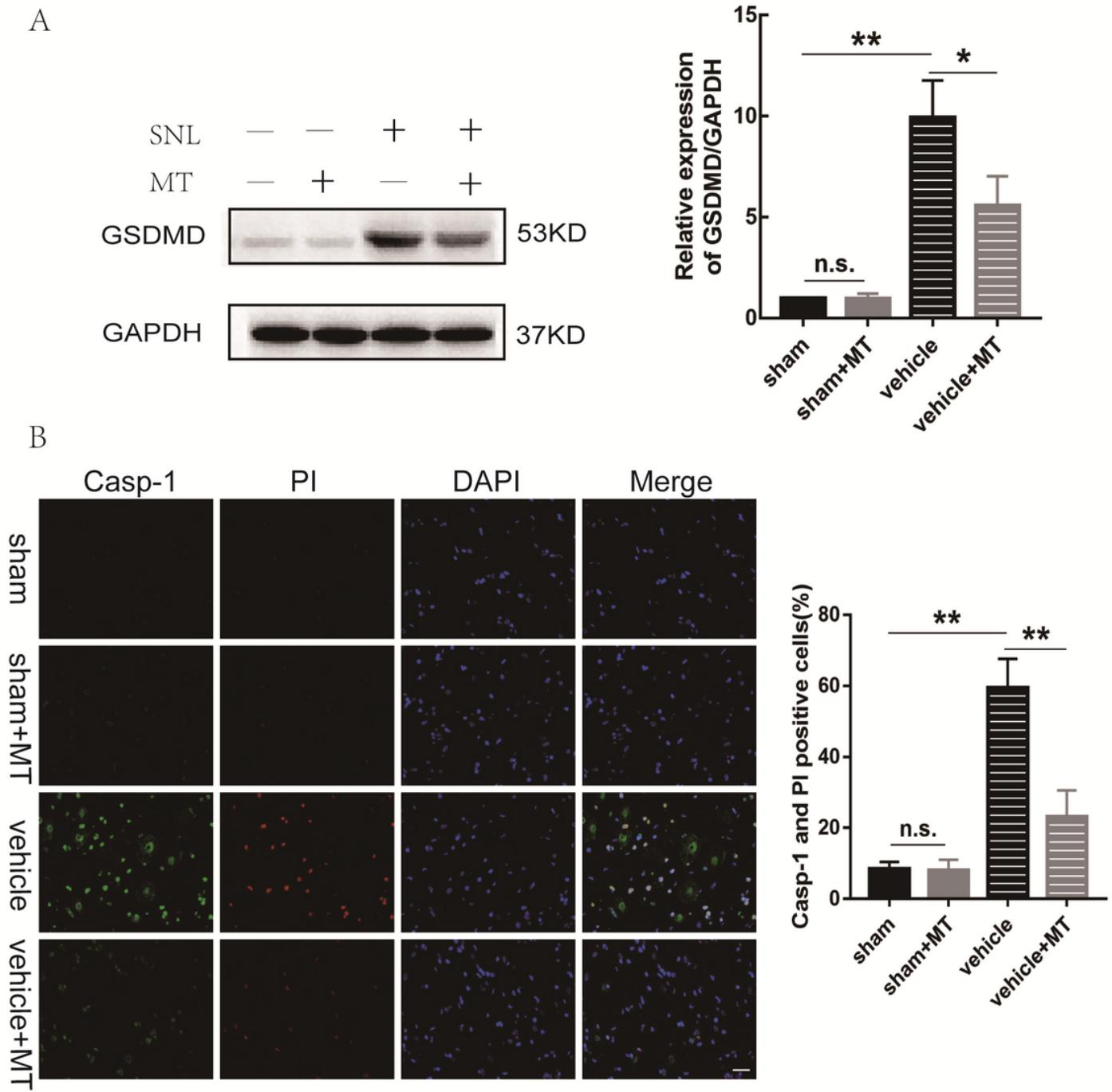


Figure 3

Melatonin prevents GSDMD activation and pyroptosis following SNL A, B SNL increased the GSDMD activation in rat which was suppressed by melatonin intraperitoneal injection ($n = 6$ /group). C Immunofluorescent labeling of the spinal cord was performed for cell pyroptosis (Green: cleaved caspase-1, Red: PI) ($n = 4$ /group). The caspase-1 and PI double-positive cells were identified as

pyroptosis. The proportion of cell pyroptosis was reduced following melatonin injection. **P < 0.01; *P < 0.05; n.s., no significant difference.

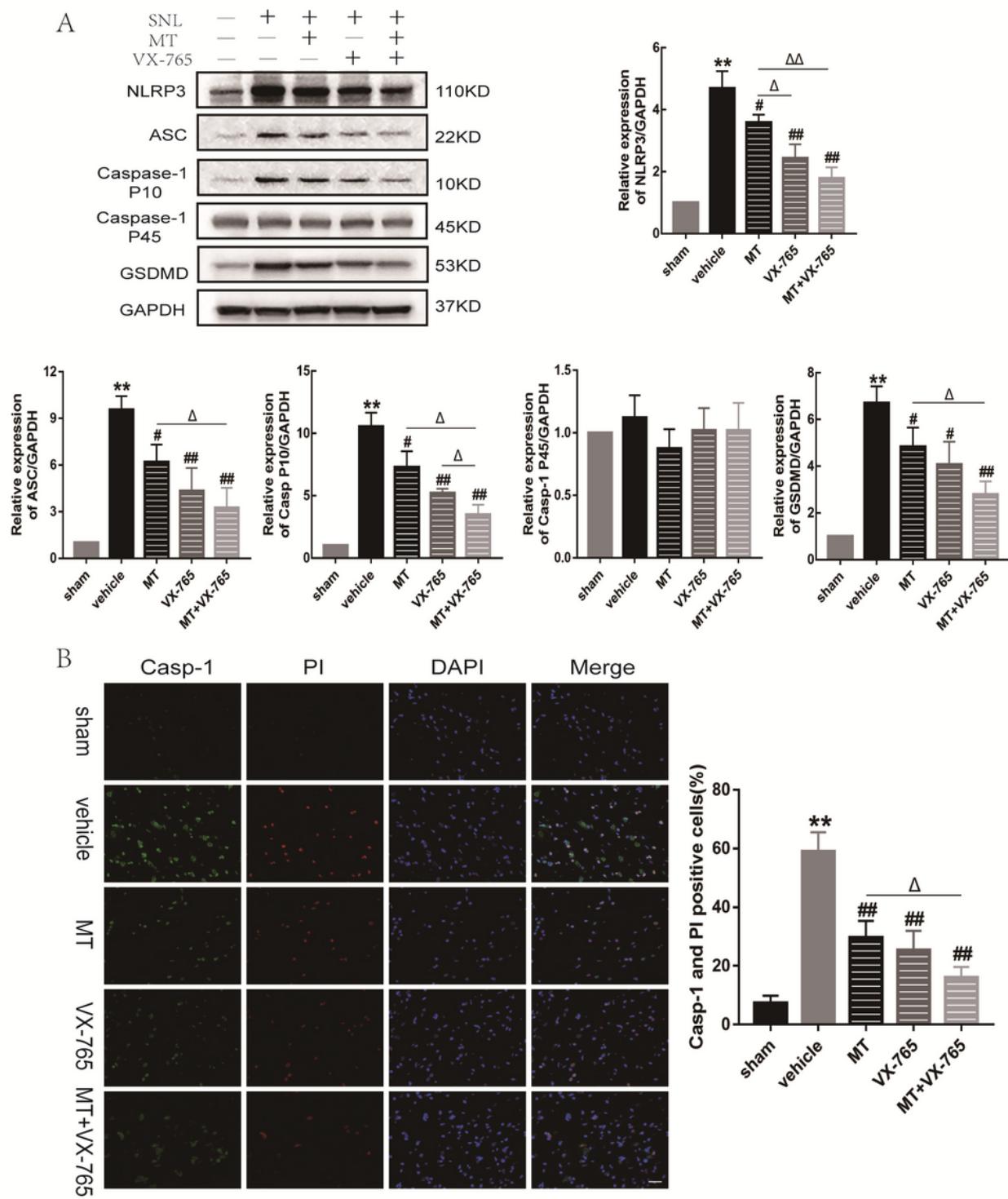


Figure 4

Caspase-1 inhibitor blunted the inflammasome activation and pyroptosis similar to the effect of melatonin. A The brand and histograms show the levels and the quantification of NLRP3, ASC, caspase-1 P45, caspase-1 P10, GSDMD, and β -actin in the spinal cords of rats from different groups respectively (n

=6/group). B Representative immunofluorescence staining showing the cell pyroptosis in the spinal cord in the different groups as indicated (n =4/group). **P < 0.01 versus the sham group; #P< 0.05 versus the vehicle group; ##P< 0.01 versus the vehicle group; △P < 0.05; △△P < 0.01; n.s., no significant difference.

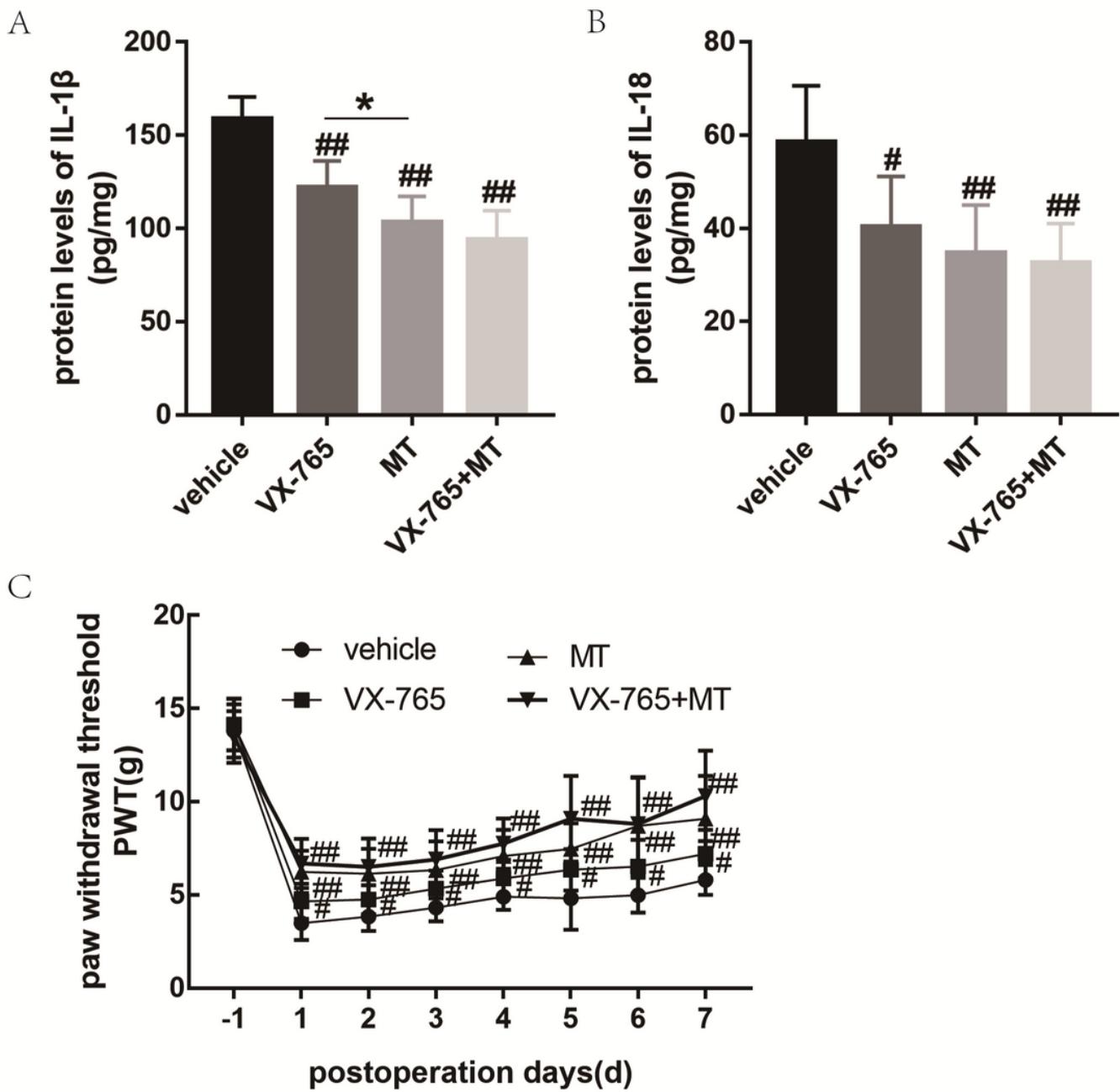


Figure 5

Effect of injection of the caspase-1-specific inhibitor VX-765 on inflammatory cytokines and SNL-induced pain hypersensitivity A, B Melatonin (A) and VX-765 (B) treatment reduced the levels of inflammatory cytokines compared with the vehicle group as measured by ELISA (n =6/group). C Behavioral analysis demonstrating the effect of melatonin and VX-765 on SNL-induced hyperalgesia (n =8/group). Melatonin

and VX-765 increased the withdrawal threshold compared to the vehicle group after surgery. #P < 0.05 versus the vehicle group; ##P < 0.01 versus the vehicle group; *P < 0.05.

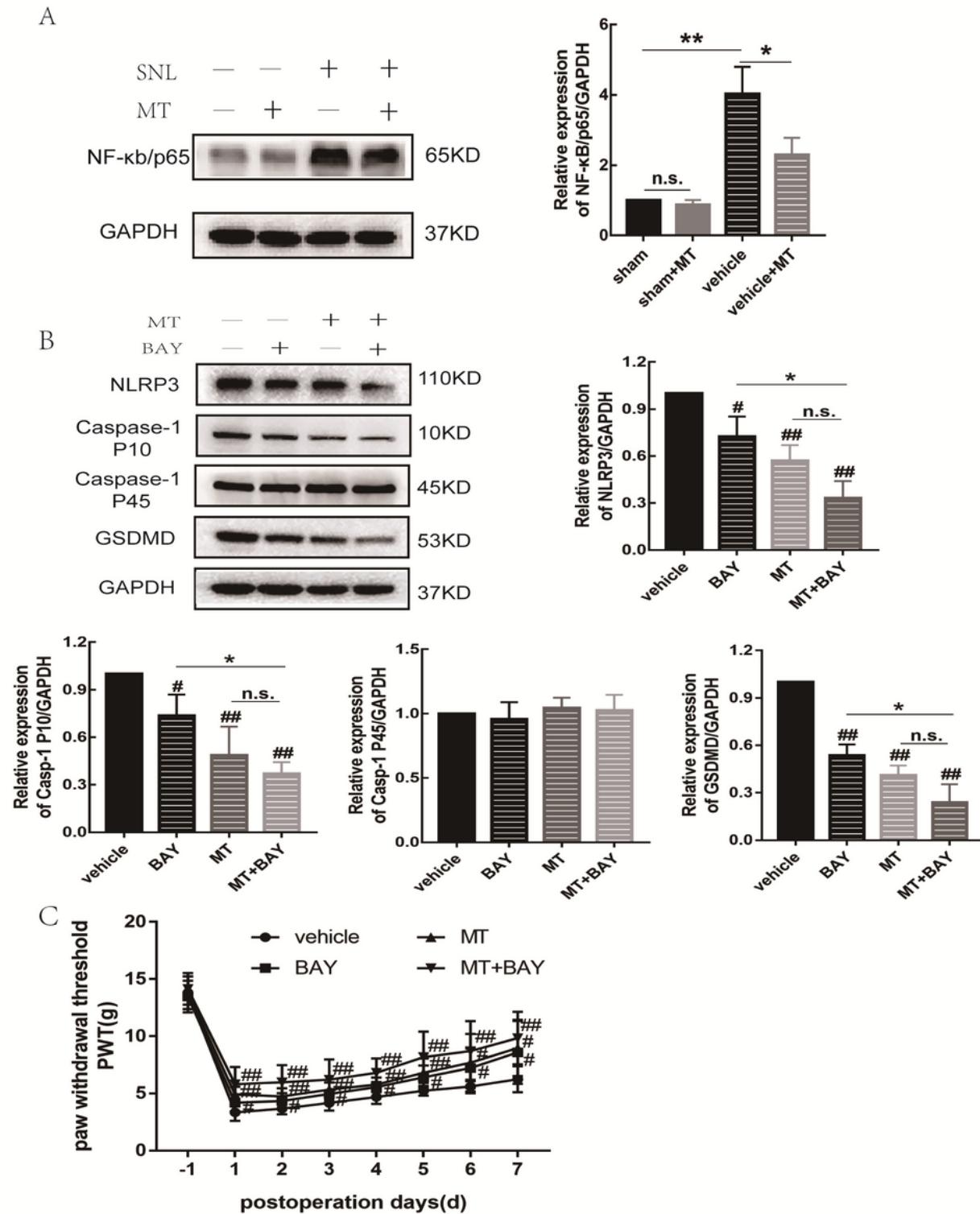


Figure 6

Melatonin prevents NF- κ B activation and effect of the NF- κ B inhibitor (BAY 11-7085) on the NLRP3 inflammasome and SNL-induced pain hypersensitivity A Suppression of overexpression of NF- κ B in spinal cord tissue in an SNL-induced rat model of NP by intraperitoneal injection of melatonin (n

BAY 11-7085 treatment (n =8/group). The injection with BAY significantly alleviated the decrease of the mechanical threshold compared with the vehicle group similar to the effect of melatonin. #P< 0.05 versus the vehicle group; ##P< 0.01 versus the vehicle group; *P < 0.05; **P < 0.01; n.s. means no significant difference. Melatonin prevents NF- κ B activation and effect of the NF- κ B inhibitor (BAY 11-7085) on the NLRP3 inflammasome and SNL-induced pain hypersensitivity A Suppression of overexpression of NF- κ B in spinal cord tissue in an SNL-induced rat model of NP by intraperitoneal injection of melatonin (n =6/group). The blot shows the expression of NF- κ B and β -actin in the different groups. The histograms show the quantification of NF- κ B normalized to β -actin in the different groups. B Western blot analysis uncovered the expression of NLRP3, ASC, caspase-1 P10, caspase-1 P45, and GSDMD using β -actin as an internal control in different groups (n =6/group). The bar chart shows the quantitative analysis of these protein levels. C Changes in mechanical allodynia induced by SNL after BAY 11-7085 treatment (n =8/group). The injection with BAY significantly alleviated the decrease of the mechanical threshold compared with the vehicle group similar to the effect of melatonin. #P< 0.05 versus the vehicle group; ##P< 0.01 versus the vehicle group; *P < 0.05; **P < 0.01; n.s. means no significant difference. Melatonin prevents NF- κ B activation and effect of the NF- κ B inhibitor (BAY 11-7085) on the NLRP3 inflammasome and SNL-induced pain hypersensitivity A Suppression of overexpression of NF- κ B in spinal cord tissue in an SNL-induced rat model of NP by intraperitoneal injection of melatonin (n =6/group). The blot shows the expression of NF- κ B and β -actin in the different groups. The histograms show the quantification of NF- κ B normalized to β -actin in the different groups. B Western blot analysis uncovered the expression of NLRP3, ASC, caspase-1 P10, caspase-1 P45, and GSDMD using β -actin as an internal control in different groups (n =6/group). The bar chart shows the quantitative analysis of these protein levels. C Changes in mechanical allodynia induced by SNL after BAY 11-7085 treatment (n =8/group). The injection with BAY significantly alleviated the decrease of the mechanical threshold compared with the vehicle group similar to the effect of melatonin. #P< 0.05 versus the vehicle group; ##P< 0.01 versus the vehicle group; *P < 0.05; **P < 0.01; n.s. means no significant difference. Melatonin prevents NF- κ B activation and effect of the NF- κ B inhibitor (BAY 11-7085) on the NLRP3 inflammasome and SNL-induced pain hypersensitivity A Suppression of overexpression of NF- κ B in spinal cord tissue in an SNL-induced rat model of NP by intraperitoneal injection of melatonin (n =6/group). The blot shows the expression of NF- κ B and β -actin in the different groups. The histograms show the quantification of NF- κ B normalized to β -actin in the different groups. B

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