

## Original Article

# Apomycin relieves neuropathic pain by attenuating NLRP3-mediated inflammasome activation via inhibiting NADPH induced ROS in microglia

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**Abstract:** Neuroinflammation in the peripheral and central nervous system plays an important role in the induction and maintenance of chronic pain. However, the potential mechanism of ROS production from inflammasome activation in microglia during neuroinflammation remains elusive. We performed spinal nerve ligation (SNL) model for chronic pain and implanted intrathecal catheter for intrathecal application of apomycin. Behavioral tests including von Frey hairs and paw withdrawal tests were constructed on postoperative days 1, 3, 5, 7, 14 and after apomycin delivery to assess mechanical threshold and heat hyperalgesia. ELISA of IL-1 $\beta$  production and real-time PCR assay of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in supernatant from microglia cells pretreatment of apomycin were performed. Besides, caspase-1 content in spinal lysis from different groups including sham-operated rats with vehicle injection, sham-operated rats with apomycin injection, SNL-injured rats with vehicle injection, SNL-injured rats with apomycin and 12 h after injection were detected by western blot analysis and also CD4 and CD8 mRNA expression were measured by Q-PCR assay. Here, we first found that apomycin significantly decreased the IL-1 $\beta$  production which was induced by NLRP3 activation in microglia cells, while the TLR4-ligation induced mRNA transcription of IL-1 $\beta$ , and other pro-inflammatory cytokines, such as IL-6 and TNF- $\alpha$  were not significantly inhibited by apomycin pretreatment. However, the activation of NF- $\kappa$ B and MAPK signaling pathway was only slightly decreased by apomycin. Furthermore, supplement of ROS in culture abolished the inhibition of IL-1 $\beta$  by apomycin. Our data suggest that apomycin ameliorates neuropathic pain by attenuating NLRP3 activation induced IL-1 $\beta$  production through inhibition of NADPH induced ROS in microglia, which outlines the novel application of apomycin in treatment of neuropathic pain and the novel potential signal pathway of apomycin.

**Keywords:** IL-1 $\beta$ , inflammasome, neuropathic pain, neuroinflammation, apomycin, ROS

## Introduction

Chronic pain is characterized by hyperalgesia, which is an increased response to noxious thermal and mechanical stimulation as well as allodynia, in which nociceptive responses occur to normally innocuous stimulation such as light touch [1-3]. Inflammatory mediators that are released locally after tissue injury can directly stimulate and cause sensitization of pain-sensing nociceptors located at nerve fibers of primary afferent neurons in peripheral tissue; these locally inflammatory mediators include classic mediators (for example, bradykinin, prostaglandins, H<sup>+</sup>, ATP and nerve growth fac-

tor), pro-inflammatory cytokines and chemokines, as well as emerging mediators such as bacterial N-formylated peptides [4-8]. Microglia cells are ubiquitously distributed throughout the brain and constantly carry out homeostatic surveillance to sense and respond to CNS abnormalities. Activation of these cells often induces increased inflammation-related molecules, such as neurotransmitters, chemokines and cytokines [9-13]. These secreted molecules form a signal network and regulate the excitability of neurons and as such an increasing number of reports regard neuropathic pain as an immune disorder affecting the neural system [9-13].

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NADPH oxidase is a multi-subunit enzyme complex responsible for the production of both extracellular and intracellular reactive oxygen species (ROS) by phagocytic cells including microglia [14-16]. NADPH oxidase is comprised of cytoplasmic subunits (p47phox, p67phox, p40phox, and Rac2), which upon phosphorylation by specific kinases can form a complex and translocate to the membrane to dock with the membrane subunits (gp91phox and p22phox) [14-16]. NADPH oxidase expression is up-regulated in microglia upon neuron injury.

The NLRP3 inflammasome is a cytosolic protein complex composed of NLRP3, ASC, and caspase-1, and assembled in response to both microbial infection and endogenous “danger signal”. The activation of NLRP3 inflammasome promotes the maturation and release of several pro-inflammatory cytokines, such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18, so it plays a critical role in the initiation of inflammation and the development of immune responses [17-19]. All danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), including ATP and particulate crystalline activators, trigger the generation of reactive oxygen species (ROS) [1, 7, 16, 20]. A ROS-dependent pathway triggers NLRP3 inflammasome complex formation. ROS blockade via chemical scavengers of ROS, pharmacological inhibitors of NADPH oxidase or siRNA-mediated knockdown of the p22phox subunit of NADPH oxidase suppressed NLRP3 activation in response to a wide range of stimulation, including ATP, *C. albicans* and various crystals (MSU, asbestos, aluminum, and hemozoin) [1, 5, 7, 16, 21]. IL-1 $\beta$  from macrophages can cause amplification of inflammation and breakdown of the blood brain barrier as well as the recruitment of immune cells while IL-1R1 $^{-/-}$  mice are resistant to EAE [22, 23]. Although NLRP3 inflammasome has been extensively investigated, its function in microglia during chronic pain still remains elusive.

Here, we find that the inhibitor of NADPH subunit gp91<sup>Phox</sup> (apomycin) ameliorates neuropathic pain by attenuating NLRP3 activation induced IL-1 $\beta$  production through inhibiting NADPH induced ROS in microglia, which outlines the novel application of apomycin in treatment of neuropathic pain and the novel target signal pathway of apomycin.

## Materials and methods

### *Animal preparation*

Male Sprague-Dawley rats (200-220 g) and C57BL/6 mice were used in these experiments in the Department of Experimental Animal Sciences, Second Military Medical University. Animals were housed with free access to food and water under a natural day/night cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second Military Medical University.

### *Primary cultures of microglia*

Microglia were isolated from primary mixed glial cell cultures prepared from newborn mice on day 14 using the ‘shaking off’ method as previously described [24]. The purity of the cultures (99%) was determined by anti-CD11b immunostaining (BD Biosciences, Franklin Lakes, NJ, USA). The cultures were maintained in Dulbecco’s modified Eagle’s minimum essential medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS, USA), 5 mg/ml bovine insulin (Sigma-Aldrich) and 0.2% glucose.

### *Spinal nerve ligation model*

The SNL (spinal nerve ligation) model was performed as described previously [25, 26]. The rats were anesthetized by 10% Chloral hydrate (0.4 ml/100 g body weight, i. p.) and placed in a prone position. An incision was made into the left of the spine at the L3-S2 level. After the left L6 vertebral transverse process was removed, the left L5 spinal nerve was exposed and tightly ligated with a 6-0 silk thread. After operation wounds were closed. Sham-operated rats underwent the same surgical procedure as the SNL rats, except that the left L5 spinal nerve was not ligated.

### *Implantation of the intrathecal catheter and drug delivery*

For spinal drug delivery, all animals received an implanted intrathecal catheter during the process of SNL surgery [25, 27, 28]. After an excision of S1 processus spinalis, a polyethylene catheter (PE-10; 20.0 cm) was inserted into the subarachnoid space at the level of the L6-S1 vertebrae until the tip of the tube (1.5 cm)

reached the lumbar cistern between the L4 and L5 vertebrae. The catheters were fixed at the lumbar part and the back of the head. Animals that exhibited motor dysfunction after an intrathecal injection of a small dose of lidocaine indicated successful implantation of the intrathecal catheter. The applied chemicals were limited at the L4-L5 DRG levels by using a small volume (10  $\mu$ l) of the injectant and limiting the rate of injection (3.3  $\mu$ l/min)<sup>17</sup>. The intrathecal application of drugs was carried out 7 days after SNL surgery. The apomycin (Santa Cruz) was dissolved in DMSO at a final concentration of 50  $\mu$ M.

### *Behavioral tests for mechanical thresholds*

Behavioral tests were conducted after SNL surgery on postoperative days 1, 3, 5, 7, 14 and after drug delivery [25, 27]. Prior to the surgery, the rats were habituated to the testing apparatus and a baseline behavior was established. A person blinded to the experiment design conducted all tests. The foot withdrawal threshold to mechanical stimulation was assessed by von Frey hairs, which were applied from underneath to the left hind-paw between the third and fourth digits (9 calibrated von Frey hairs with bending forces of 0.4-15.0 g). For each test, the rats were placed in a plastic chamber (20 $\times$ 20 $\times$ 25 cm) and habituated for at least 30 min. The chamber was placed on top of a mesh screen so that mechanical stimulation could be administered to the plantar surface of the left hind paw. The same set of von Frey stimulation was given in an ascending order. Each von Frey hair was tested repetitively 10 times with a 2-3 min interval between stimulation and the bending force that evoked 50% paw withdrawal occurrence was deemed as the mechanical threshold. If the 15 g failed to evoke withdrawal response, the value was recorded as 15 g. A decrease of 7 g in paw withdrawal mechanical threshold indicated mechanical hyperalgesia.

### *Behavioral tests for heat hyperalgesia*

Heat hyperalgesia was detected using the paw withdrawal test [25, 27]. The rat was placed under an inverted clear plastic chamber cage (20 $\times$ 18 $\times$ 13 cm) on an elevated glass floor (3 mm thick). After an adaption period of 30 min, heat hyperalgesia was determined using a projector lamp bulb (automatic plantar analgesia taster, Institute of Biomedical Engineering,

Chinese Academy of Medical Science, Tianjin, China), which provided a radiant heat source aiming through the glass onto the mid-plantar hind paw. The latency of the withdrawal reflex was used as the pain threshold. A latency of 20 s was used to avoid tissue damage. Tests were performed five times for each paw, with a 5-min interval between each test. Rats were tested for their baseline response twice before surgery. All behavioral tests were performed blinded by one person.

### *Real-time PCR*

Total RNAs from L5 DRGs were isolated using an Ultra spec RNA isolation kit (Biotecx). Triplicate real-time PCR samples were preceded as previously reported on the ABI Prism 7000 sequence detection system. The primers for TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were according to previously reported [29]. The primers for rat CD4 and CD8 were as following: (CD4F 5'-ATGTGC-CGAGGCTTCTCTTTCAG; CD4R 5'-TAACTTGTTCTTATATCCCAGAA; CD8F 5'-ATGGCCTCACGGGTGATCTGCTT; CD8R 5'-CTGGAGGAGTTCGGAGCTGGAGT), which produced 210 bp length of amplification. All real-time RT-PCR experiments were performed at least three times, and the mean  $\pm$  SEM values are presented.

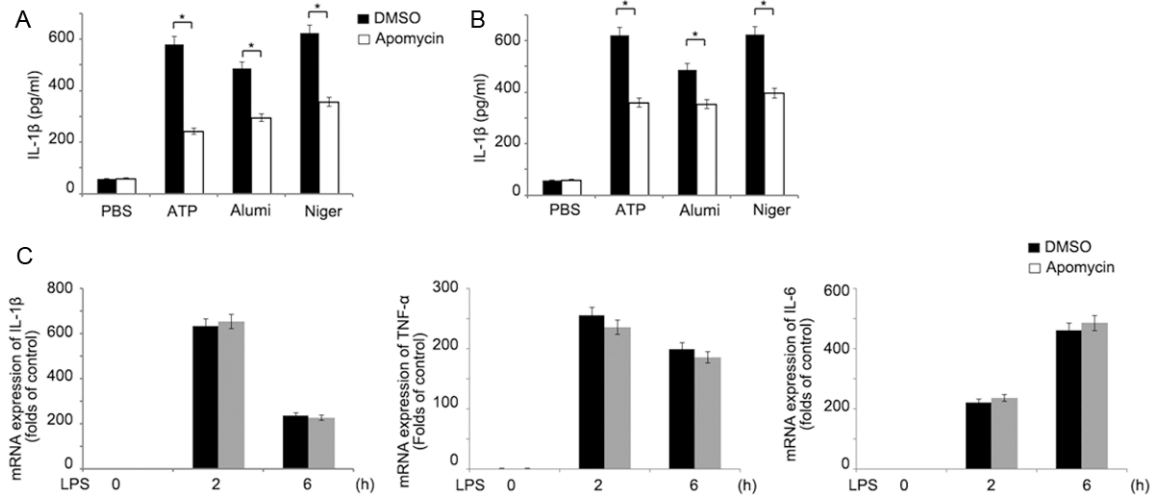
### *Immunoblot assay*

Immunoblot assay was performed as previously reported [29]. Briefly, Cells pretreated as described above were lysed with RIPA buffer (Cell Signaling Technology, Beverly, MA) supplemented with protease inhibitor cocktail. Protein concentrations of the extracts were measured with BCA assay. Anti-Caspase-1 p45&p20 (C4851) and anti- $\beta$ -actin were from Sigma-Aldrich. Anti-murine Caspase-1 p45&p20 (Casper-1) was from AdipoGen. Anit-phosphor-p65 and anit-phosphor-c-jun were from Cell Signaling Technology.

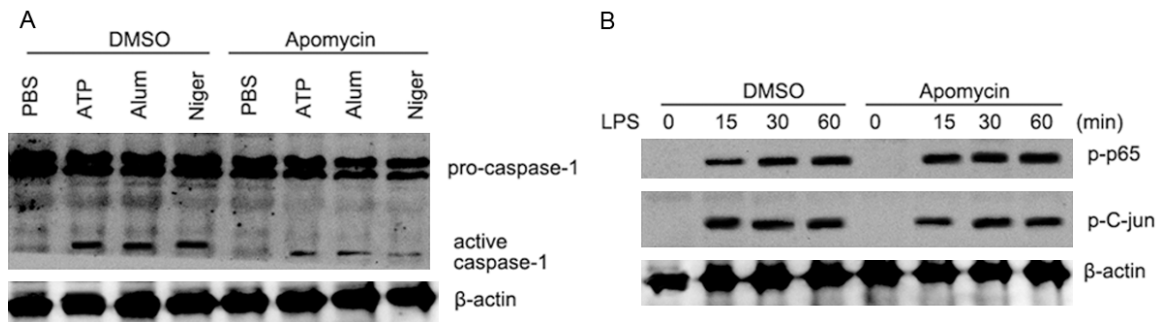
### *ELISA*

The production of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  was measured using supernatant from  $1 \times 10^6$  microglia cells or lysis from 100 mg/well of whole spinal homogenate with a commercial enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems (Minneapolis, MN), as described previously [29, 30]. Nigericin (10  $\mu$ m), ATP (1 mM), Aluminum salts (200  $\mu$ g/ml) were from Sigma-Aldrich.

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**Figure 1.** Apomycin inhibited NLRP3 activation induced IL-1 $\beta$  production. (A) ELISA of IL-1 $\beta$  production in supernatant from microglia cells pretreatment of apomycin, then primed with LPS for 6 h and re-stimulation with ATP, aluminum or nigericin toxin for 0.5 h. (B) ELISA of IL-1 $\beta$  production in supernatant from microglia cells primed with LPS for 6 h, and then treated of apomycin and re-stimulated with ATP, aluminum or nigericin toxin for 0.5 h. (C) Q-PCR assay of IL-1 $\beta$ , TNF- $\alpha$  and IL-6 mRNA expression in cells as (A). Data are representative of three different experiments. Error bars represent mean  $\pm$  SEM. \* $P < 0.01$ .



**Figure 2.** Apomycin inhibited NLRP3 signaling activation. (A) Western blot analysis of caspase-1 activation in microglia cells primed with LPS for 6 h, and then treatment of apomycin and re-stimulation with ATP, aluminum or nigericin toxin for 0.5 h. (B) Western blot analysis of NF- $\kappa$ B transcription factor p65 and MAPK transcription factor c-jun activation in microglia cells pretreated with apomycin and then re-stimulated with LPS as indicated. Data are representative of three different experiments.

## Statistical analysis

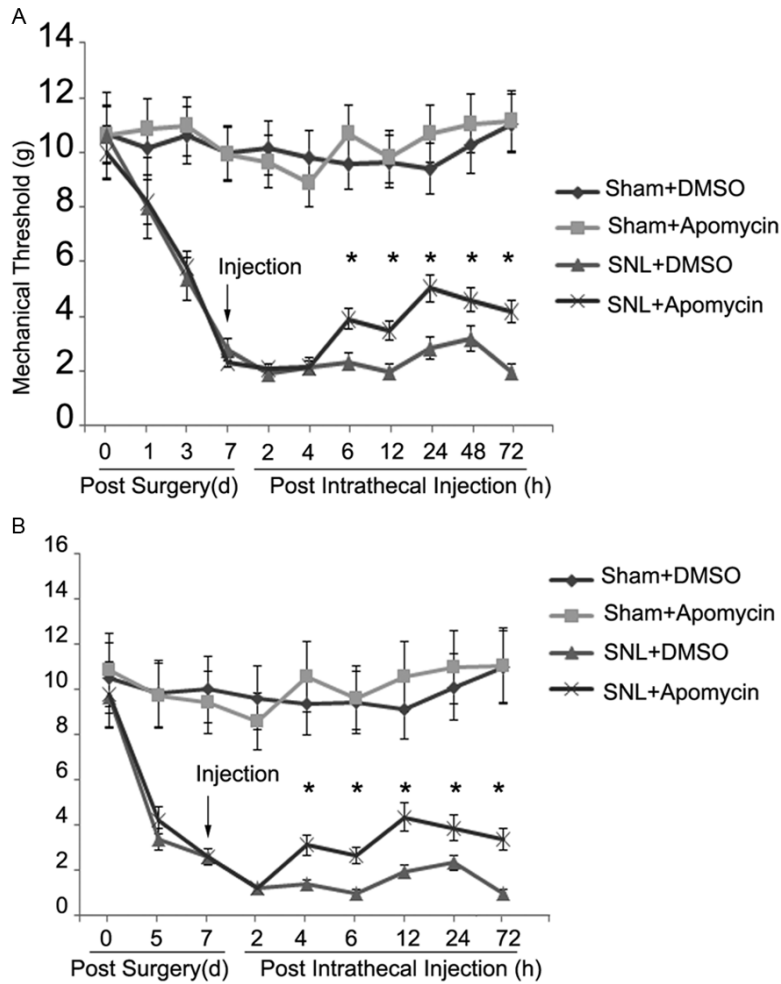
Data are represented as mean  $\pm$  SEM. The statistical significance of differences was analyzed using the PASW statistical program (SPSS Inc.) by performing ANOVA between the three groups and SNK test between two groups. For behavior responses, a two-way ANOVA with repeated measure analyses of variance was performed followed by the holm-sidak post hoc test for multiple comparisons. A value of  $\alpha=0.05$  was used as significant level, and a  $P$  value of  $<0.05$  was considered statistically significant.

## Results

### Apomycin significantly inhibited NLRP3 activation induced IL-1 $\beta$ production

As the function of NADPH and its production ROS in inflammasome activation in microglia is unknown, we used the classical NLRP3 inflammasome activation model by stimulating LPS primed microglia with ATP, aluminum and nigericin toxin. We first found that the LPS stimulation induced ROS production was significantly inhibited by apomycin pretreatment (data not

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**Figure 3.** Intrathecal injection of apomycin attenuates SNL-induced mechanical allodynia and heat hyperalgesia 7 days after surgery. (A) Mechanical allodynia was measured using the sham-operated rats with vehicle injection (Sham+DMSO, n=8), sham-operated rats with apomycin injection (Sham+Apomycin, n=8, 50  $\mu$ M), SNL-injured rats with vehicle injection (SNL+DMSO, n=8), SNL-injured rats with apomycin and (SNL+Apomycin, n=8, 50  $\mu$ M) 12 h after injection. (B) Heat hyperalgesia was measured using rats the same to (A). Data are representative of three different experiments. Error bars represent mean  $\pm$  SEM. \* $P < 0.001$ , compared to SNL+DMSO group.

shown), which is in accordance with previously reported. The production of IL-1 $\beta$  was significantly inhibited by pretreatment of apomycin before LPS priming (Figure 1A). Apomycin could also inhibit the IL-1 $\beta$  production even after LPS priming (Figure 1B), which indicated that apomycin may directly inhibit the NLRP3 inflammasome activation, but not inhibit the TLR4 signaling. Accordingly, TLR4-ligation induced mRNA transcription of pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$  was not inhibited significantly by apomycin pretreatment (Figure 1C). Thus, apomycin may directly

inhibit the NLRP3 inflammasome activation.

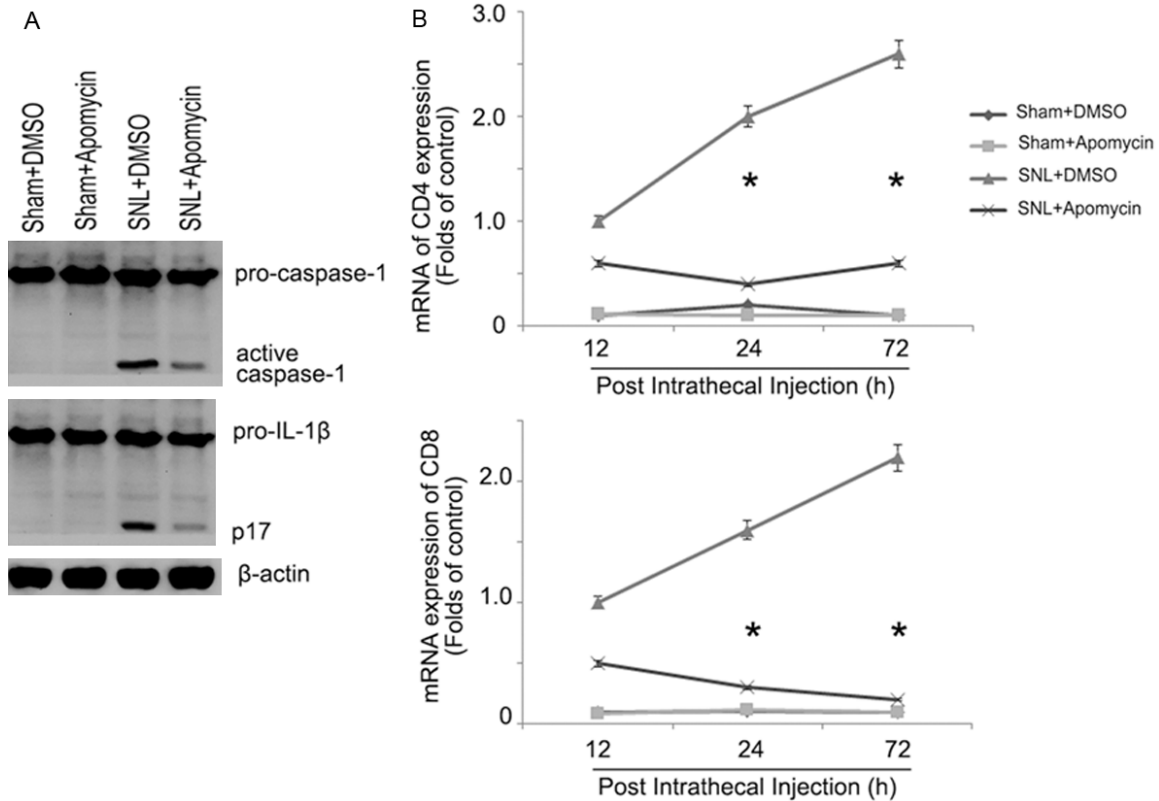
### Apomycin inhibited NLRP3 signal activation

To further confirm that apomycin may directly inhibit the NLRP3 inflammasome activation, the activation of caspase-1 and cleavage of IL-1 $\beta$  was detected by immunoblot. The cleavage of caspase-1 and IL-1 $\beta$  was both inhibited by pretreatment of apomycin (Figure 2A). The activation of p65 and *c-jun*, two vital transcripts of NF- $\kappa$ B and MAPK in TLR signaling pathway, was only slightly inhibited by apomycin pretreatment (Figure 2B). These data indicate that apomycin inhibits of IL-1 $\beta$  production by inhibiting capsase-1 activation.

### Intrathecal apomycin administration reduces SNL-induced mechanical allodynia and heat hyperalgesia

Chronic apomycin treatment attenuates beta amyloid plaque size and microglial number in mice [31]. To address whether inhibition of IL-1 $\beta$  production by apomycin is able to relieve allodynia, apomycin was given to the rats through an intrathecal catheter 1 d after SNL surgery. One group of mice received intrathecal injection of apomycin at doses of 50  $\mu$ M, and the other group received equal volume of vehicle injection as a control. Then we tested the withdrawal threshold for mechanical pain after intrathecal injection of apomycin. The SNL-induced mechanical allodynia was significantly attenuated in the 50  $\mu$ M group 4 hours after injection, this effect last longer than 72 hours (Figure 3A). In addition, vehicle or apomycin treatment in sham-operated rats did not show any effect on mechanical allodynia.

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**Figure 4.** Intrathecal injection of apomycin attenuates SNL-induced neuroinflammation. (A) Western blot analysis of caspase-1 in spinal lysate from rats sham-operated rats with vehicle injection (Sham+DMSO), sham-operated rats with apomycin injection (Sham+Apomycin, 50  $\mu$ M), SNL-injured rats with vehicle injection (SNL+DMSO), SNL-injured rats with apomycin and (SNL+Apomycin, 50  $\mu$ M) 12 h after injection. (B) Q-PCR assay of CD4 and CD8 mRNA expression in spinal lysate from rat treated as (A) and collected samples as indicated time after injection. Data are representative of three different experiments. Error bars represent mean  $\pm$  SEM. \* $P < 0.001$ , compared to SNL+DMSO group.

Then we measured thermal hyperalgesia of the SNL rats treated intrathecally with apomycin (50  $\mu$ M) or vehicle. Apomycin (50  $\mu$ M) significantly blocked heat hyperalgesia 4 hours after injection. Sham-operated rats intrathecally injected with apomycin or vehicle were not significantly different from the baseline latencies (Figure 3B). These data indicate that inhibition of IL-1 $\beta$  production by apomycin reduces SNL-induced mechanical allodynia and heat hyperalgesia.

### *Intrathecal apomycin administration reduces SNL-induced pro-inflammatory cell infiltration*

To determine whether the anti-hyperalgesia mechanism of apomycin is related to the stimulus of the IL-1 $\beta$  production in SGC-7901 cells, we measured the expression of caspase-1 activation and IL-1 $\beta$  p17 production 12 h after treating with apomycin. We observed a potent

decrease of caspase-1 activation and IL-1 $\beta$  p17 production in both the injured and contralateral DRG of apomycin treated group compared with the control group (Figure 4A). Then to further explore whether apomycin also inhibits the sequentially neuroinflammation, CD8 and CD4 expression was determined by Q-PCR. CD8 and CD4 expression, which indicates the CD8 and CD4 positive T cells infiltration into intrathecal space, was also significantly inhibited by apomycin treatment (Figure 4B). These data indicated that inhibition of IL-1 $\beta$  production by apomycin prevented further amplification of inflammation.

### Discussion

Emerging studies have shown microglial-neuronal interaction plays a critical role in the genesis of pathological pain and alteration of glial functions is a culprit causing aberrant neuronal

activities in the pain related signaling pathway. In present study, we find that the inhibitor of NADPH subunit gp91<sup>Phox</sup> (apomycin) ameliorates neuropathic pain by attenuating NLRP3 activation induced IL-1 $\beta$  production through inhibiting NADPH induced ROS in microglia, which outlines the novel application of apomycin in treatment of neuropathic pain and the novel targeting signal pathway of apomycin.

Although acute inflammation produces transient central sensitization, chronic pain is associated with a long-lasting and sometimes even permanent central sensitization that persists after acute inflammation has been resolved. For example, Toll-like receptor 4 (TLR4) expressed in the spinal cord was shown to mediate the transition from acute to persistent mechanical hypersensitivity after the resolution of inflammation in a rodent model of arthritis, in which the danger-associated molecular patterns (DAMPs) play critical function [27, 32]. During neuron injury, DAMPs, such as HMGB-1, mammalian double stranded DNA and uric acid crystals, released by injured cells and activate an inflammatory response via TLR4, NOD or NLRP3 signaling. Pain and inflammation can also be dissociated in other conditions; for example, periodontal disease (which occurs as a result of chronic inflammation) is not associated with pain [27, 32].

NLRP3 inflammasome-mediated IL-1 $\beta$  production is a two-step process: a primary signal that induces pro-IL-1 $\beta$  and NLRP3 synthesis and a secondary signal that activates the inflammasome and the subsequent caspase-1 processing. The first step is accomplished by activation of TLR4, which also induces expression of TNF- $\alpha$  in microglia during pain by activation of NF- $\kappa$ B signaling. Caspase-1 activation is the key step in inflammasome activation [21, 23, 33]. The activation of NLRP3 in microglia during pain remains unknown. Here, we demonstrate that gp91<sup>phox</sup> inhibitor apomycin can inhibit caspase-1 activation and thus decreases IL-1 $\beta$  production and decrease the sequential inflammation amplification, which adds new light to the application and function of inflammasome during pain.

The mitochondrial ROS detector MT-Red showed that ROS are generated in the mitochondria of dorsal horn neurons. Production of mitochondrial ROS by intrathecal injection of

the electron transport complex inhibitors antimycin A and rotenone in normal mice resulted in mechanical hyperalgesia [34]. Our data show that phagocytic ROS also share an important part in pain and inhibition of this phagocytic ROS can also ameliorate pain.

Taking our *in vivo* and *in vitro* results together, we suggest that inhibition of phagocytic ROS production in microglia by apomycin ameliorates neuropathic pain behavior after SNL by blunting NLRP3 activation induced IL-1 $\beta$  production, which outlines the novel application of apomycin in treatment of neuropathic pain and the novel target signal pathway of apomycin.

### Acknowledgements

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### Disclosure of conflict of interest

None.

### Abbreviations

NLRP3, NACHT domain-, leucine-rich repeat-, and pyrin domain containing protein 3; NF- $\kappa$ B, Nuclear Factor Kappa B; MAPK, Mitogen activated protein kinase; LPS, lipopolysaccharide; ROS, reactive oxygen species; SNL, spinal nerve ligation.

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