Ginsenoside Rh2 Ameliorates Neuropathic Pain by inhibition of the miRNA21-TLR8-mitogen-activated protein kinase axis

Yuan-Yuan Fu1,2, Jian-Ke Cen1, Hao-Lin Song2, Si-Yuan Song1, Zhi-Jun Zhang2, and Huan-Jun Lu1

Abstract
Ginsenoside Rh2 is one of the major bioactive ginsenosides in Panax ginseng. Although Rh2 is known to enhance immune cells activity for treatment of cancer, its anti-inflammatory and neuroprotective effects have yet to be determined. In this study, we investigated the effects of Rh2 on spared nerve injury (SNI)-induced neuropathic pain and elucidated the potential mechanisms. We found that various doses of Rh2 intrathecal injection dose-dependently attenuated SNI-induced mechanical allodynia and thermal hyperalgesia. Rh2 also inhibited microglia and astrocyte activation in the spinal cord of a murine SNI model. Rh2 treatment inhibited SNI-induced increase of proinflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-1 and IL-6. Expression of miRNA-21, an endogenous ligand of Toll like receptor (TLR)8 was also decreased. Rh2 treatment blocked the mitogen-activated protein kinase (MAPK) signaling pathway by inhibiting of phosphorylated extracellular signal-regulated kinase expression. Finally, intrathecal injection of TLR8 agonist VTX-2337 reversed the analgesic effect of Rh2. These results indicated that Rh2 relieved SNI-induced neuropathic pain via inhibiting the miRNA-21-TLR8-MAPK signaling pathway, thus providing a potential application of Rh2 in pain therapy.

Keywords
Ginsenoside Rh2, spared nerve injury, Chronic pain, miRNA-21, TLR8

Introduction
Ginseng (Panax ginseng C.A. Meyer) is a traditional Oriental herbal drug widely distributed in East Asia. Ginsenosides as the main active constituents have various pharmacological activities, such as anti-depressive,1 antipruritic,2 anti-inflammatory,3 antiallergic4 and anticancer5 activities. Previous phytochemical and pharmacological investigations have demonstrated the antinociceptive effects of ginseng extracts in various pain models including those of abdominal,6 neuropathic,7 chronic8 and incisional9 pain. Several different mechanisms of action have been suggested to explain these effects, including reduced neural hypersensitivity, antagonism of adrenergic activation, and inhibition of microglial activation.10–12

Ginsenoside Rh2 is one of the major bioactive ginsenosides in P. ginseng, and has especially been used in the treatment of cancer.5 In terms of structure, Rh2 can be divided into S-type and R-type configurations, among which 20(S)-Rh2 monomer is the main configuration isolated from medical herbs and plays a major role in anticancer activity.13 (Figure 1). Previous studies have revealed that ginsenoside Rh2 significantly inhibited...
lipopolysaccharide (LPS)-induced activation of BV2 cells and decreased production of inflammatory mediators via modulating the transforming growth factor-β1/Smad pathway. However, the potential effect of Rh2 on neuropathic pain has not been investigated, and its mechanisms of action remain largely unknown.

Toll-like receptors (TLRs) are reported to play a critical role in the innate and adaptive immune responses. After binding with ligands, TLRs initiate and regulate the inflammatory response via the release of cytokines. TLR8 belongs to the TLR family and is important for viral single-stranded RNA recognition, neurite outgrowth and immune cell regulation. Our previous study demonstrated that TLR8 was located in the endoplasmic reticulum, endosomes, and lysosomes of dorsal root ganglion (DRG) neurons, which was activated by endogenous miRNA-21 and contributed to the pathogenesis of spinal-nerve-injury-induced neuropathic pain. Ginsenoside Rh2 was previously reported to mediate miRNA expression in human non-small cell lung cancer cells. Thus, this study aimed to explore the effects of Rh2 on spared nerve injury (SNI)-induced neuropathic pain. Our results demonstrated that Rh2 specifically attenuated miRNA-21 to inhibit TLR8 activation and relieve nociceptive pain in SNI mice. Also, the anti-inflammatory effect of Rh2 was achieved by inhibiting the production of inflammatory cytokines through blocking the mitogen-activated protein kinase (MAPK) signaling pathways. This study provided a potential medicinal application of Rh2 in pain therapy.

Materials and Methods

Experimental animals and treatments

Adult male ICR mice (age: 8 weeks, weighing 26–30 g) were provided by the Experimental Animal Center at Nantong University. Tlr8−/− mice were developed by Cyagen (Suzhou, China). All experiments were approved by the Animal Care and Use Committee of Nantong University. Mice were kept in each cage in a 12-h light-dark cycle with free access to food and water. Animal treatments were performed in accordance with the guidelines of the International Association for the Study of Pain.

SNI-induced neuropathic pain model

The neuropathic pain model was induced by SNI. The operation was performed as described by Decosterd and Woolf. After disinfecting the surgical site with alcohol and iodine, careful blunt dissection was performed through the biceps femoris muscle to expose the sciatic, sural, tibial and common peroneal nerves. The tibial and common peroneal nerves were tightly ligated with 6.0 silk suture and then completely severed in between, leaving the sural nerve intact. After transection, the muscle fascia and skin were sutured.

Drugs and administration

Ginsenoside Rh2 (Figure 1) was purchased from Shanghai Source Leaf Biology Co. Ltd. and dissolved in 0.01 M phosphate-buffered saline (PBS) containing 0.4% dimethyl sulfoxide, with an intrathecal injection concentration of 100 μmol. VTX-2337 was purchased from Active Biochem (Hong Kong, China) with an intrathecal injection concentration of 100 ng. Intrathecal-injection was made with a 30 G needle between the L5 and L6 intervertebral spaces to deliver the reagents to the cerebrospinal fluid. Rh2 (100 μL) or 10 μL VTX-2337 was injected intrathecally.

Pain behavior analysis

The von Frey test was performed to measure paw mechanical sensitivity. The mice were put in boxes on an elevated metal mesh floor and allowed 30 min for habituation before the examination. The plantar surface of the hind paw was stimulated with a series of von Frey hairs with logarithmically incrementing stiffness (0.02–2.56 g, North Coast). The 50% paw withdrawal threshold was determined using Dixon’s up-down method.

The Hargreaves test was used to detect paw thermal sensitivity. The mice were put in a plastic box placed on a glass plate, and the plantar surface was exposed to a beam of radiant heat through a transparent glass surface. The baseline latencies were adjusted to 10–14 s with a maximum of 20 s as a cutoff to prevent potential injury. All the behavioral experimenters were done by individuals that were blinded to the treatment or genotypes of the mice.

RNA isolation and quantitative real-time polymerase chain reaction for mRNAs and microRNAs

Total RNA from L4 or L5 DRG was extracted using a Total RNA Extraction Kit (Easy-spin™, Intron, USA) and reverse transcribed into cDNAs using GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA). Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and GAPDH were amplified by using DreamTaq DNA Polymerases (Thermo-Fisher, Waltham, MA, USA). For miRNA detection, small RNAs were extracted using the RNAiso kit (Takara, Shiga,
Japan), and 10 ng small RNA was reverse transcribed into cDNA using the One Step PrimeScript microRNA cDNA Synthesis kit (Takara). GAPDH and U6 small nuclear RNA were used as endogenous controls to normalize differences for mRNA and miRNA detection, respectively. Melt curves were performed upon completion of the cycles to ensure that nonspecific products were absent. Quantification was performed by normalizing target gene cycle threshold (Ct) values with corresponding GAPDH Ct (mRNA) or U6 Ct (miRNA), and then analyzed with the $2^{-\Delta\Delta Ct}$ method. The sequences of primers are shown in Table 1.

**Immunofluorescence**

For tissue immunofluorescence staining,27 the spinal cord and L4–L6 DRG were dissected from 8-week-old ICR mice. The mice were killed at 1 h after drug injection. The samples were fixed with 4% paraformaldehyde and frozen in Tissue-Tek O.C.T. The samples were cut into 15-µm thick sections. The cryosections were incubated with blocking buffer [4% bovine serum albumin (BSA) in PBS with 0.01% Triton X100] for 2 h and with polyclonal Iba-1 (rabbit, 1:1,000; Wako/Fuji, Richmond, VA, USA), GFAP Forward CCAAGATGAAACCAACCTGA (mouse, 1:2,000; Millipore, Burlington, MA, USA), c-Fos (rabbit, 1:1,000; Abcam, Cambridge, UK), or phosphorylated extracellular signal-regulated kinase (pERK) (rabbit, 1:5,000; Abcam) antisera overnight at 4°C. After being washed for 15 min, the samples were incubated with the secondary antibodies Donkey anti-mouse 488 (1:1,000; Jackson ImmunoResearch, West Grove, PA, USA), Donkey anti-rabbit 488 (1:1,000; Jackson ImmunoResearch). The fluorescence signals were checked and captured under a fluorescence microscope (Eclipse Ni-E). The fluorescence images were analyzed with ImageJ (NIH, Bethesda, MD, USA). Analysis methods for fluorescence images have been described previously.28 After selecting the fluorescence regions and choosing no fluorescence area as the background, ImageJ automatically generated the immunohistochemistry index. The number of c-Fos-positive neurons in the dorsal horn of the spinal cord ipsilateral to the SNI surgery site was counted and then analyzed with the $2^{-\Delta\Delta Ct}$ method. The sequences of primers are shown in Table 1.

**In situ hybridization**

Expression of miR-21 in DRG was determined by using the mature mouse miRNA detection probe as previously reported.29 DRG sections were treated with proteinase K (BosterBio, Pleasanton, CA, USA) for 2 min at room temperature, and then washed in RNase-free PBS. After digestion with proteinase, sections were post-fixed with the 1% paraformaldehyde/PBS and washed in diethyl-pyrocarbonate-treated ultrapure water. After prehybridization and hybridization, sections were washed with 2× saline sodium citrate (SSC), 0.5× SSC and 0.2× SSC buffers. To identify the colocalization of miR-21 and neuronal nuclei (NeuN), the above sections under miR-21 in situ hybridization (ISH) were incubated overnight at 4°C with primary antibodies against NeuN. On the following day, secondary antibody was added and incubated for 2 h. The signal was detected with a fluorescence microscope (Eclipse Ni-E).

**Western blotting**

Western blotting was performed as described previously.30 After the mice were killed and perfused with saline intracardially at 1 h after drug injection, the L4–L6 DRG were homogenized in lysis buffer containing phosphatase and protease inhibitors (Sigma-Aldrich, St Louis, MO, USA). The protein concentration was measured by BCA Protein Assay (Thermo Fisher Scientific). Protein samples (30 μg) were loaded in each lane of SDS-PAGE, and transferred to polyvinylidene difluoride membranes. After blocking with 5% BSA solution, the membranes were incubated with primary antibody against pERK or ERK (rabbit, 1:1,000; Cell Signaling Technology, Danvers, MA, USA), and GAPDH (mouse, 1:10,000; Millipore). The membranes were further incubated with secondary antibody (goat-anti-rabbit, 1:1,000; LI-COR, Lincoln, NE, USA) and images were detected by Odyssey CLx system (Odyssey, LICOR, USA). The intensity of bands was statistically analyzed by ImageJ.

**Statistics**

Data are shown as mean ± SEM. The behavioral data were analyzed by two-way ANOVA. Differences between groups were compared using one-way ANOVA or Student’s t-test. $p < 0.05$ was considered statistically significant.

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**Table 1.** The list of primer sequences designed for quantitative real-time RT-PCR.

| Gene   | Primers         | Sequences (5’–3’)       |
|--------|----------------|-------------------------|
| TNF-α  | Forward         | GTTCTATGGCCAGACCCCTCAC  |
|        | Reverse         | GCCAACACTAGTTGTTGCTTTTG |
| IL-1β  | Forward         | TCCAGGATAGGACATGACAC    |
|        | Reverse         | GAAGCTCACACCAGGCTATA    |
| IL-6   | Forward         | CCACCTCACAAGTGCGAGCTTA  |
|        | Reverse         | CCAGTTTTGGATGATCCATTTC  |
| miR-21 | Forward         | TAGCTTTACAGCTAGTGT      |
|        | Reverse         | GGCCCAAGCGGAGAAGTGTGTTT |
| U6     | Forward         | GCTTCGGAAGCACATATAACTAA |
|        | Reverse         | CGAATTTGCGTGTACTCCTT    |
| Gapdh  | Forward         | AAATGTTGAAGTGCGTGTGAC   |
|        | Reverse         | CAAACATCTCAGCTTTGCCACTG |
| Iba-1  | Forward         | ATGAGCCAAAGCGAGATT      |
|        | Reverse         | CTTCAAGTTTGGAGGCGAG     |
| GFAP   | Forward         | CCAAGATGAAACCCACCTGGA   |
|        | Reverse         | TCCAGCGATTCACCCTTTC     |
Results

Ginsenoside Rh2 attenuates SNI-induced pain

To test the antinociceptive effect of Rh2 in SNI-induced chronic pain, we first tested the effect of single intrathecal injection of Rh2 at a high concentration, but it did not affect neuropathic pain (data not shown). Then, we injected different doses of Rh2 daily for 6 consecutive days (Figure 2(a)). The development of mechanical allodynia and thermal hyperalgesia was evaluated for 10 days after surgery (Figure 2(b) and (c)). SNI significantly induced mechanical allodynia by decreasing the mechanical stimulus threshold from day 1 after surgery (Figure 2(b)). Intrathecal administration of Rh2 significantly attenuated mechanical allodynia at 100 μM but not at 1 μM (Figure 2(b)). Rh2 also attenuated SNI-induced thermal hyperalgesia. The antinociceptive effect of Rh2 continued until 10 days after SNI surgery (Figure 2(c)). These results suggested that Rh2 could relieve SNI-induced mechanical allodynia and thermal hyperalgesia.

Ginsenoside Rh2 inhibits spinal microglia and astrocyte activation in the SNI model

Glia cells in the spinal cord dorsal horn (SCDH) play important roles in the development and maintenance of chronic neuropathic pain. Therefore, we proceeded to explore the effect of Rh2 on astrocytic and microglial activation in SCDH of SNI model mice after drug (or vehicle) continuous treatment 6 days. We checked immunoactivity of glial fibrillary acidic protein (GFAP; an astrocyte marker) and ionized calcium binding adaptor molecule 1 (Iba-1; a microglia marker) in SCDH of vehicle or Rh2 treatment groups. We found strong increases in Iba-1 (or GFAP) immunoreactivity (Figure 3(a), (b), (f), (g)) and the soma area (Figure 3(c), (h)) of Iba-1 (or GFAP) positive cells after SNI. However, Rh2 treatment significantly attenuated Iba-1 (or GFAP) immunoreactivity (Figure 3(b), (g)). Quantification of morphological parameters showed that compared to controls, SNI surgery increased the microglia in soma area and reduced the process length in the SCDH, which was reversed by intrathecal Rh2 (Figure 3(c), (d)), but not for astrocytes (Figure 3(h), (i)). qPCR of Iba-1 and GFAP confirmed the expression levels of Iba-1 and GFAP after SNI and decrease by Rh2 treatment (Figure 3(e), (j)). These results indicated that Rh2 treatment suppressed microglial and astrocytic activation to attenuate pain hypersensitivity induced by SNI.

Inhibitory effect of ginsenoside Rh2 on release of proinflammatory cytokines in the SNI model

TNF-α, IL-1β and IL-6 are important proinflammatory cytokines in mediating peripheral sensitization and neuropathic pain. The release of proinflammatory cytokines was increased after SNI. To check whether the antinociceptive effect of Rh2 was associated with downregulation of proinflammatory cytokines, we measured expression of TNF-α, IL-1β and IL-6 in DRG after Rh2 continuous treatment 6 days. qPCR showed
that mRNA expression of these proinflammatory cytokines in the Rh2 treatment group was lower than in the vehicle group (Figure 4). These results indicated that the antinociceptive effect of Rh2 may act by preventing the expression of proinflammatory cytokines.

**Ginsenosides Rh2 attenuates miRNA-21 increase in DRG after SNI**

The miRNA-21 was reported to be secreted from tumors as a ligand to TLR8. Our previous study revealed that miRNA-
Ginsenoside Rh2 relieves neuropathic pain by suppressing TLR8 activation

Because Rh2 can suppress miRNA-21 expression, we confirmed the effect of Rh2 on TLR8 activation. Continuous intrathecal injection of Rh2 inhibited mechanical allodynia and hyperalgesia in SNI model mice. However, intrathecal injection of TLR8 agonist VTX-2337 reversed the analgesic effect of Rh2 but not in TLR8 knockout mice.
These data confirmed that Rh2 attenuates SNI-induced pain hypersensitivity through inhibition of TLR8 activation.

**Ginsenoside Rh2 inhibits SNI-induced pERK and c-Fos activation in DRG**

We further determined whether the analgesic effects of Rh2 were associated with inhibition of the pERK signaling pathway. We used western blotting to check the expression of phosphorylation of ERK in DRG. Western blotting showed that expression of pERK was significantly increased after SNI surgery; however, Rh2 treatment decreased SNI-induced pERK upregulation (Figure 7(a) and 7(b)). We also used immunofluorescence to confirm this result, which was consistent with the western blotting results (Figure 7(c)–(e)). The c-Fos is encoded by the proto-oncogene c-fos and has been extensively used as a marker for neuronal activity in various regions including in the spinal cord.36 We therefore sought to assess the change in neuronal activity of dorsal horn neurons of the L4–L5 spinal cord using c-Fos immunohistochemistry. c-Fos immunoreactivity was observed throughout the ipsilateral dorsal horn of the lumbar spinal cord after SNI (Figure 7(f)). However, the number of c-Fos-positive neurons in the dorsal horn was significantly lower in Rh2 treated mice (Figure 7(g)), which suggested that Rh2 reduced SNI-induced neural hyperactivity through inhibition of the MAPK signaling pathway.

**Discussion**

In the current study, we found a novel effect of Rh2 on neuropathic pain and its antinociceptive mechanism. Our results demonstrated that intrathecal administration of Rh2 relieved SNI-induced mechanical allodynia and thermal hyperalgesia. In addition, Rh2 administration attenuated SNI-induced microglia and astrocyte activation in the spinal cord and reduced miRNA-21 expression. Finally, we also found that Rh2 reduced SNI-induced neural hyperactivity through inhibition of the MAPK signaling pathway.

Previous studies have shown that ginsenoside Rh2 had anti-inflammatory and anticancer activities and acts against atopic dermatitis by modulating T helper type 2 differentiation.37,38 However, the role of Rh2 in neuropathic pain has not yet been studied. We first demonstrated that repeated intrathecal injection of Rh2 attenuated SNI-induced thermal hyperalgesia and mechanical allodynia. These findings indicated that Rh2 had analgesic properties.

The Toll like receptor (TLR) family is a group of 13 members with various physiological as well as pathological functions (such as mediating innate immunity).39 Recent research has investigated the functional role of TLRs in pain and itch.40 Several TLRs are expressed in DRG neurons and glial cells under chronic pain conditions. TLR2 and TLR4 modulate glial activation in the spinal cord and contribute to the development of neuropathic pain.41 TLR3 is expressed in small nociceptive DRG neurons, and regulates sensory neuronal excitability and spinal synaptic transmission.42 TLR7 is co-expressed with transient receptor potential ankyrin subtype 1 protein (TRPA1)
in DRG neurons and involved in both pain and itch processes. TLR8 is thought to affect inflammation and neuronal apoptosis. Our previous research also showed that TLR8 was mostly expressed in small DRG neurons and mediated spinal-nerve-injury-induced neuropathic pain. Here, intrathecal injection of VTX, an agonist of TLR8, induced pain hypersensitivity in mice, which was inhibited by Rh2 administration, suggesting that the antinociceptive effect of Rh2 may be mediated by TLR8 inhibition.

Under the condition of nerve injury, activation of glial cells leads to the development of neuroinflammation, which is responsible for induction and maintenance of chronic pain. We found that Rh2 reduced microglial and astrocytic activation induced by SNI. Besides, when the microglia and astrocytes received pain signals, inflammatory factors such as TNF-α, IL-1β and IL-6 were secreted to maintain or develop the pathology of pain. TNF-α, IL-1β and IL-6, which are proinflammatory cytokines, have been implicated in

Figure 7. Intrathecal injection of Rh2 inhibits pERK and c-fos activation. (a and b) pERK expression of SNI model mice treated or untreated with Rh2. pERK expression was increased in DRG after SNI surgery, and inhibited by Rh2. n=3 for each group, The Unpaired t-test was conducted for two group comparisons. *p < 0.05. (c) Immunostaining of pERK in the DRG; (d) Statistical data show the pERK staining intensity in the DRG, n = 3 for each group, The Unpaired t-test was conducted for two group comparisons. ***p < 0.001. (e) Co-expression of pERK and a neuronal marker, β-tublin (Tuj1) in the DRG at 6 days after SNI. (f) Immunostaining of c-Fos in the SDH; (d) Statistical data show the number of pERK positive neurons, n = 3 for each group, The Unpaired t-test was conducted for two group comparisons. ***p < 0.001. Scale bar=100 μm.
neuropathic pain. Our results showed that expression of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 was significantly increased in DRG neurons after SNI and inhibited by Rh2 treatment. IL-1\(\beta\) expression is increased in TRPV1-positive DRG neurons following peripheral neuroinflammation. IL-6 is upregulated in DRG neurons or spinal cord following nerve-injury-induced neuropathic pain. Therefore, Rh2 may relieve SNI-induced allodynia and hyperalgesia through inhibition of neuroinflammation mediated by TNF-\(\alpha\), IL-1\(\beta\) and IL-6.

The miRNAs are small, noncoding RNAs, 19–24 nt in length. They can negatively regulate gene expression by canonical binding to their target mRNAs or direct interaction with proteins. Recent array studies have shown that nerve injury changes expression of a variety of miRNAs in the DRG. Some miRNAs are thought to modulate nociception. For example, miR-let7b has a pronociceptive effect via mediation of neuron–neuron cross-excitation. miR-let7b is released by DRG neurons and activates TRPA1 channels to depolarize sensory neurons. miRNA-21 is also upregulated in ipsilateral DRG neurons after peripheral nerve injury, which is associated with ipsilateral mechanical hypersensitivity. Downregulation of miRNA-21 prevents the development of ipsilateral mechanical hypersensitivity and decreases the number of inflammatory macrophages in DRG.

Consistent with other previous studies, we also found an increase in miRNA-21 expression in DRG neurons of SNI model mice. As an endogenous ligand of TLR8, miRNA-21 bound to TLR8 in DRG neurons and activated downstream pathways to cause neuroinflammation. Yeung et al. found that miRNA-21 secreted by exosomes can be transferred from one cell to another. Thus, we also do not exclude miRNA-21 secretion from nearby neurons. Ginsenoside Rh2 is reported to regulate miRNAs in different kinds of disease models. Wu et al. found that expression of miRNA-21 in several human glioma cell lines such as U251, T98MG and A172 was decreased after treatment with ginsenoside Rh2. An et al. also showed that ginsenoside Rh2 downregulated miRNA-21 in human non-small cell lung cancer A549 cells as part of its anticancer mechanism. In the current study, we found a significant decrease in miRNA-21 in DRG neurons and relief of pain hypersensitivity after intrathecal treatment with Rh2. The possible antinociceptive mechanisms of Rh2 may be because Rh2 can downregulate miRNA-21 that is induced by nerve injury, and this further inhibits TLR8 activation to relieve pain.

MAPKs, including c-Jun N-terminal kinase, p38 MAPK and ERK, are a family of serine/threonine protein kinases that transduce extracellular stimuli into intracellular post-
translational transcriptional responses. A variety of extracellular stimuli activate intracellular MAPks by phosphorylation, which modulates the intracellular responses that drive different downstream signaling. Nerve injury induces activation of ERK. In this study, we observed upregulation of pERK expression in SNI and alleviation of neuropathic pain by Rh2 was accompanied with downregulation of pERK, suggesting that the analgesic effects of Rh2 may correlate with inactivation of the MAPK signaling pathway.

In summary, the current findings supported the idea that Rh2 suppresses TLR8 activation through inhibition of miRNA-21 after SNI. The anti-inflammatory effect of Rh2 was achieved by inhibiting production of inflammatory cytokines through blocking the MAPK signaling pathway (Figure 8). Future studies will focus on how Rh2 modulates miRNA-21 and other possible pathways in nociceptive regulation. As Rh2 is involved in SNI-induced pain regulation, our study provided valuable information for its clinical application. Collectively, our findings suggested that Rh2 may exert its analgesic effect through inhibition of the miRNA21–TLR8–MAPK signaling axis.

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Author contributions
Yuan-Yuan Fu and Hao-Lin Song performed RT-PCR and animal behaviors, Jian-Ke Chen and Si-Yuan Song performed immuno-staining and Western-blot and data analysis. Huan-Jun Lu initiated, designed the study. Huan-Jun Lu and Zhi-Jun Zhang wrote the manuscripts.

Declaration of conflicting interests
The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORCID iD
Huan-Jun Lu https://orcid.org/0000-0001-6200-1437

References
1. Zheng M, Xin Y, Li Y, Xu F, Xi X, Guo H, Cui X, Cao H, Zhang X, Han C. Ginsenosides: A Potential Neuroprotective Agent. Biomed Res Int 2018; 2018: 8174345.
2. Trinh HT, Shin YW, Han SJ, Han MJ, Kim DH. Evaluation of antipruritic effects of red ginseng and its ingredients in mice. Planta Med 2008; 74(3): 210–214.
3. Im DS. Pro-Resolving Effect of Ginsenosides as an Anti-Inflammatory Mechanism of Panax ginseng. Biomolecules 2020; 10(3).
4. Park EK, Choo MK, Kim EJ, Han MJ, Kim DH. Antiinflammatory activity of ginsenoside Rh2. Biol Pharm Bull 2003; 26(11): 1581–1584.
5. Li X, Chu S, Lin M, Gao Y, Liu Y, Yang S, Zhou X, Zhang Y, Hu Y, Wang H, Chen N. Anticancer property of ginsenoside Rh2 from ginseng. Eur J Med Chem 2020; 203: 112627.
6. Wang CZ, Yao H, Zhang CF, Chen L, Wan JY, Huang WH, Zeng J, Zhang QH, Liu Z, Yuan J, Bi Y, Sava-Segal C, Du W, Xu M, Yuan C. American ginseng microbial metabolites attenuate DSS-induced colitis and abdominal pain. Int Immunopharmacol 2018; 64: 246–251.
7. Lee JY, Choi HY, Park CS, Kim DH, Yune TY. Total saponin extract, ginsenoside Rb1, and compound K alleviate peripheral and central neuropathic pain through estrogen receptors on rats. Phytother Res 2021; 35(4): 2119–2132.
8. Lorz LR, Kim MY, Cho JY. Medicinal potential of Panax ginseng and its ginsenosides in atopic dermatitis treatment. J Ginseng Res 2020; 44(1): 8–13.
9. Kim MK, Kang H, Baek CW, Jung YH, Woo YC, Choi GJ, Shin HY, Kim KS. Antinociceptive and anti-inflammatory effects of ginsenoside Rf in a rat model of incisional pain. J Ginseng Res 2018; 42(2): 183–191.
10. Kim HJ, Jung SW, Kim SY, Cho IH, Kim HC, Rhim H, Kim M, Nah SY. Panax ginseng as an adjuvant treatment for Alzheimer’s disease. J Ginseng Res 2018; 42(4): 401–411.
11. Kim UI, Park CH, Lee SH, Yoon MH. The role of spinal adrenergic receptors on the antinoceptive effect of ginsenosides in a rat postoperative pain model. Korean J Anesthesiol 2013; 65(1): 55–60.
12. Qi D, Zhu Y, Wen L, Liu Q, Qiao H. Ginsenoside Rg1 restores the impairment of learning induced by chronic morphine administration in rats. J Psychopharmacol 2009; 23(1): 74–83.
13. Gao H, Liang D, Li C, Xu G, Jiang M, Li H, Yin J, Song Y. 2-Deoxy-Rh2: A novel ginsenoside derivative, as dual-targeting anti-cancer agent via regulating apoptosis and glycolysis. Biomed Pharmacother 2020; 124: 109891.
14. Vinoth Kumar R, Oh TW, Park YK. Anti-Inflammatory Effects of Ginsenoside-Rh2 Inhibits LPS-Induced Activation of Microglia and Overproduction of Inflammatory Mediators Via Modulation of TGF-beta1/Smad Pathway. Neurochem Res 2016; 41(5): 951–957.
15. Komi DEA, Mortaz E, Amani S, Tiotiu A, Folkerts G, Adcock IM. The Role of Mast Cells in LeE-Independent Lung Diseases. Clin Rev Allergy Immunol 2020; 58(3): 377–387.
16. Liu T, Ji RR. Toll-Like Receptors and Itch. In: Carstens E, Akiyama T (eds). Itch: Mechanisms and Treatment. Boca Raton FL: CRC Press, 2014.
17. Li L, Liu X, Sanders KL, Edwards JL, Ye J, Si F, Gao A, Huang L, Hsueh EC, Ford DA, Hoft DF, Peng G. TLR8-Mediated
Metabolic Control of Human Treg Function: A Mechanistic Target for Cancer Immunotherapy. Cell Metab 2019; 29(1): 103–123.

18. Ma Y, Haynes RL, Sidman RL, Vartanian T. TLR8: an innate immune receptor in brain, neurons and axons. Cell Cycle 2007; 6(23): 2859–2868.

19. Martinez-Espinoza I, Guerrero-Plata A. The Relevance of TLR8 in Viral Infections. Pathogens 2022; 11(2): 134.

20. Zhang JZ, Guo JS, Li SS, Wu XB, Cao DL, Jiang BC, Jing PB, Bai XQ, Li CH, Wu ZH, Lu Y, Gao Y. TLR8 and its endogenous ligand miR-21 contribute to neuropathic pain in murine DRG. J Exp Med 2018; 215(12): 3019–3037.

21. An IS, An S, Kwon KJ, Kim YJ, Bae S. Ginsenoside Rh2 mediates changes in the microRNA expression profile of human non-small cell lung cancer A549 cells. Oncol Rep 2013; 29(2): 523–528.

22. Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 2000; 87(2): 149–158.

23. Gabriel AF, Marcus MA, Walenkamp GH, Joosten EA. The CatWalk method: assessment of mechanical allodynia in experimental chronic pain. Behav Brain Res 2009; 198(2): 477–480.

24. Gonzalez-Cano R, Boivin B, Bullock D, Cornelissen L, Andrews N, Costigan M. Up-Down Reader: An Open Source Program for Efficiently Processing 50% von Frey Thresholds. Front Pharmacol 2018; 9: 433.

25. Cheah M, Fawcett JW, Andrews MR. Assessment of Thermal Pain Sensation in Rats and Mice Using the Hargreaves Test. Bio Protoc 2017; 7(16): e2506.

26. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988; 32(1): 77–88.

27. Im K, Mareninov S, Diaz MFP, Yong WH. An Introduction to Performing Immunofluorescence Staining. Methods Mol Biol 2019; 1897: 299–311.

28. Jensen EC. Quantitative analysis of histological staining and fluorescence using ImageJ. Anat Rec (Hoboken) 2013; 296(3): 378–381.

29. Friedman TN, Yousuf MS, Catuneanu A, Desai M, Juzwik CA, Fournier AE, Kert BJ. Profiling the microRNA signature of the peripheral sensory ganglia in experimental autoimmune encephalomyelitis (EAE). J Neuroinflammation 2019; 16(1): 223.

30. Hirano S. Western blot analysis. Methods Mol Biol 2012; 926: 87–97.

31. Inoue K, Tsuda M. Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential. Nat Rev Neurosci 2018; 19(3): 138–152.

32. Ji RR, Donnelly CR, Nedergaard M. Astrocytes in chronic pain and itch. Nat Rev Neurosci 2019; 20(11): 667–685.

33. Takahashi J, Ebara S, Kamimura M, Kinoshita T, Misawa H, Shimogata M, Tozuka M, Takaoka K. Pro-inflammatory and anti-inflammatory cytokine increases after spinal instrumentation surgery. J Spinal Disord Tech 2002; 15(4): 294–300.

34. Hung AL, Lim M, Doshi TL. Targeting cytokines for treatment of neuropathic pain. Scand J Pain 2017; 17: 287–293.

35. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, Lovat F, Fadda P, Mao C, Nuovo GJ, Zanesi N, Crawford M, Ozer GH, Wernicke D, Alder H, Caligiuri MA, Nana-Sinkam P, Perrotti D, Croce CM. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. Proc Natl Acad Sci U S A 2012; 109(31): E2110–E2116.

36. Bullitt E. Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. J Comp Neurol 1990; 296(4): 517–530.

37. Ko E, Park S, Lee JH, Cui CH, Hou J, Kim MH, Kim SC. Ginsenoside Rh2 Ameliorates Atopic Dermatitis in NC/Nga Mice by Suppressing NF-kappaB-Mediated Thymic Stromal Lymphopoietin Expression and T Helper Type 2 Differentiation. Int J Mol Sci 2019; 20(24): 6111.

38. Li LC, Piao HM, Zheng MY, Lin ZH, Choi YH, Yan GH. Ginsenoside Rh2 attenuates allergic airway inflammation by modulating nuclear factor-kappaB activation in a murine model of asthma. Mol Med Rep 2015; 12(5): 6946–6954.

39. Fitzgerald KA, Kagan JC. Toll-like Receptors and the Control of Immunity. Cell 2020; 180(6): 1044–1066.

40. Liu T, Gao YJ, Ji RR. Emerging role of Toll-like receptors in the control of pain and itch. Neurosci Bull 2012; 28(2): 131–144.

41. Rocha Sobrinho HMD, Silva DJD, Gomides LF, Dorta ML, Oliveira MAP, Ribeiro-Dias F. TLR4 and TLR2 activation is differentially associated with age during Parkinson’s disease. Immuno Invest 2018; 47(1): 71–88.

42. Kostoula C, Shaker T, Cerovic M, Craparotta I, Marchini S, Butti E, Pascente R, Iori V, Garlanda C, Aronica E, Martino G, Ravizza T, Carmant L, Vezzani A. TLR3 preconditioning induces anti-inflammatory and anti-ictogenic effects in mice mediated by the IRF3/IFN-beta axis. Brain Behav Immun 2019; 81: 598–607.

43. Kampo S, Cui Y, Yu J, Anahab TW, Falagan AA, Bayor MT, Wen QP. Scorpion Venom peptide, AGAP inhibits TRPV1 and potentiates the analgesic effect of lidocaine. Heliyon 2021; 7(12): e08560.

44. Tang SC, Yeh SJ, Li Y, Wang YC, Baik SH, Santro T, Widiapradja A, Manzanero S, Sobey CG, Jo DG, Arumugam TV, Jeng J. Evidence for a detrimental role of TLR8 in ischemic stroke. Exp Neurol 2013; 250: 341–347.

45. Kuzumaki N, Narita M, Takaoka K. Pro-inflammatory factors and anti-inflammatory factors in innate immune responses. J Exp Med 2019; 215(12): 3019–3037.
Experimental Verification. *Evid Based Complement Alternat Med* 2020; 2020: 2545806.

48. Stavast CJ, Erkeland SJ. The Non-Canonical Aspects of MicroRNAs: Many Roads to Gene Regulation. *Cells* 2019; 8(11): 1465.

49. Park CK, Xu ZZ, Berta T, Han Q, Chen G, Liu XJ, Ji RR. Extracellular microRNAs activate nociceptor neurons to elicit pain via TLR7 and TRPA1. *Neuron* 2014; 82(1): 47–54.

50. Wu XB, Jing PB, Zhang ZJ, Cao DL, Gao MH, Jiang BC, Gao YJ. Chemokine receptor CCR2 contributes to neuropathic pain and the associated depression via increasing NR2B-mediated currents in both D1 and D2 dopamine receptor-containing medium spiny neurons in the nucleus accumbens shell. *Neuropsychopharmacology* 2018; 43(11): 2320–2330.

51. Simeoli R, Montague K, Jones HR, Castaldi L, Chambers D, Kelleher JH, Vicca V, Pitcher T, Grist J, Al-Ahdaal H, Wong L, Perretti M, Lai J, Mouritzen P, Heppenstall P, Malcangio M. Exosomal cargo including microRNA regulates sensory neuron to macrophage communication after nerve trauma. *Nat Commun* 2017; 8(1): 1778.

52. Au Yeung CL, Co NN, Tsuruga T, Yeung TL, Kwan SY, Leung CS, Li Y, Lu ES, Kwan K, Wong KK, Schmandt R, Lu KH, Mok SC. Exosomal transfer of stroma-derived miR21 confers paclitaxel resistance in ovarian cancer cells through targeting APAF1. *Nat Commun* 2016; 7: 11150.

53. Lu DZ, Dong W, Feng XJ, Chen H, Liu JJ, Wang H, Zang LY, Qi MC. CaMKII(delta) regulates osteoclastogenesis through ERK, JNK, and p38 MAPKs and CREB signalling pathway. *Mol Cell Endocrinol* 2020; 508: 110791.