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ORIGINAL ARTICLE

Activation of Double-Stranded RNA–Activated Protein Kinase in the Dorsal Root Ganglia and Spinal Dorsal Horn Regulates Neuropathic Pain Following Peripheral Nerve Injury in Rats

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Abstract

Double-stranded RNA (dsRNA)–activated kinase (PKR) is an important component in inflammation and immune dysfunction. However, the role of PKR in neuropathic pain remains unclear. Here, we showed that lumbar 5 spinal nerve ligation (SNL) led to a significant increase in the level of phosphorylated PKR (p-PKR) in both the dorsal root ganglia (DRG) and spinal dorsal horn. Images of double immunofluorescence staining revealed that p-PKR was expressed in myelinated A-fibers, unmyelinated C-fibers, and satellite glial cells in the DRG. In the dorsal horn, p-PKR was located in neuronal cells, astrocytes, and microglia. Data from behavioral tests showed that intrathecal (i.t.) injection of 2-aminopurine (2-AP), a specific inhibitor of PKR activation, and PKR siRNA prevented the reductions in PWT and PWL following SNL. Established neuropathic pain was also attenuated by i.t. injection of 2-AP and PKR siRNA, which started on day 7 after SNL. Prior repeated i.t. injections of PKR siRNA prevented the SNL-induced degradation of IκBα and IκBβ in the cytosol and the nuclear translocation of nuclear factor κB (NF-κB) p65 in both the DRG and dorsal horn. Moreover, the SNL-induced increase in interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) production was diminished by this treatment. Collectively, these results suggest that peripheral nerve injury–induced PKR activation via NF-κB signaling–regulated expression of proinflammatory cytokines in the DRG and dorsal horn contributes to the pathogenesis of neuropathic pain. Our findings suggest that pharmacologically targeting PKR might be an effective therapeutic strategy for the treatment of neuropathic pain.

Keywords Double-stranded RNA–activated kinase · Spinal nerve ligation · NF-κB · Proinflammatory cytokine · Neuropathic pain

Introduction

Neuropathic pain caused by a lesion or disease of the somatosensory nervous system is a common chronic pain condition with a major impact on quality of life [1]. Peripheral nerve injury and inflammation are common causes of neuropathic pain. Following nerve injury, different classes of primary sensory fibers exhibit phenotypic changes in epitopes in their dorsal root ganglion (DRG), including abnormal expression of ion channels and receptors and increased production of proinflammatory mediators, thereby resulting in enhanced excitability in injured and neighboring uninjured primary afferent neurons [2, 3]. These changes lead to a persistent increase in pain-related synaptic transmission in the spinal dorsal horn, which is referred to as central sensitization and is a critical event in the development and

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maintenance of neuropathic pain [4, 5]. An increasing number of studies have demonstrated that nerve injury–induced changes in epitopes in the DRG and dorsal horn depend on the activation of different intracellular signal pathways. The roles of protein kinase A (PKA), protein kinase B (PKB), protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and many other signaling pathways in neuropathic pain have been verified by different studies in recent decades [1].

Double-stranded RNA (dsRNA)–activated protein kinase (PKR) is a well-established Ser/Ther protein kinase that is activated by dsRNA during viral infection [6, 7]. The canonical function of PKR is to inhibit global protein synthesis by phosphorylating the alpha subunit of eukaryotic initiation factor 2 (eIF2) in response to dsRNA, thus preventing viral proliferation [6]. In addition to its established antiviral and anti-tumor activities, recent studies have demonstrated that PKR contributes to inflammation and immune regulation through several signaling pathways [8, 9]. It has been shown that in addition to dsRNA, PKR is activated by multiple stimuli, such as tumor necrosis factor-α (TNF-α), lipopolysaccharides (LPS), and chemotherapy- and irradiation-induced DNA damage, through a mechanism involving autophosphorylation. Active PKR mediates the activation of MAPKs, inhibitors of κB (IκB) kinase (IKK), and IFN-β–promoter simulator 1 (IPS-1) signaling and then affects diverse transcription factors, including interferon regulatory factor 3 (IRF3), nuclear factor κB (NF-κB), **c-Jun** N-terminal kinases (JNKs), and activating transcription factor 2 (ATF2), which are required for the expression of genes encoding proinflammatory cytokines [8]. Previous studies have revealed that PKR is involved in the LPS-induced activation of signal transducer and activator of transcription 1 (STAT1) inflammatory signaling in glial cells in the rat brain [10] and contributes to the development of Alzheimer's disease in patients [11]. Functional inhibition of PKR in the hippocampus suppressed Nod-like receptor protein 1 (NLRP1) inflammasome activation and effectively attenuated chronic constriction injury (CCI)–induced depression-like behaviors [12]. This finding suggests that the activation of PKR is involved in the development of neuroinflammation in the nervous system.

Neuropathic pain has been associated with excessive inflammation in both the peripheral and central nervous systems, which may contribute to the initiation and maintenance of persistent pain [13–18]. However, the expression and activation of PKR in the dorsal root ganglia and spinal cord and the role of PKR activation in neuropathic pain following peripheral nerve injury remain to be examined. Therefore, in the current study, we explored the role of PKR activation in mechanical allodynia and thermal hyperalgesia induced by lumbar 5 spinal nerve ligation (SNL) in male rats. We found that SNL increased the level of phosphorylated PKR in the L4/5 DRG and dorsal horn, and knockdown or pharmacological inhibition of PKR attenuated neuropathic pain by repressing nuclear factor κB (NF-κB) signaling.

Materials and Methods

Animal Preparation

A total of 236 adult male Sprague Dawley rats weighing 250–300 g were included in this study. The animals were randomly divided into different experiments as shown in Fig. 1. Animals were housed in separate cages under a 12/12 h light/dark cycle at a room temperature of 23 ± 1 °C with food and water ad libitum. The animals were purchased from the Laboratory Animal Center of Zhengzhou University, Zhengzhou, China. All procedures conformed to the guidelines of the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committee of Zhengzhou University in China. Efforts were made to minimize the number of animals used and their suffering.

Lumbar 5 Spinal Nerve Ligation (SNL)

The SNL was carried out in rats to induce neuropathic pain following the procedures described by Kim and Chung [19] and our published paper [20, 21]. Briefly, an incision on the lower back midline was made after the animals were anesthetized with isoflurane (2–3%) vaporized through a nose cone. The S1 lumbar transverse process was identified and then removed with the rongeur. The underlying L5 spinal nerve was isolated and ligated with a 3–0 silk thread. The ligated nerve was then transected distal to the ligature. The wound was washed with saline and closed in layers (fascia and skin) with 3–0 silk thread. In sham-operated rats, the left L5 spinal nerve was isolated, but without ligation.

Intrathecal Catheterization and Drug Delivery

Drugs were delivered intrathecally. The intrathecal catheterization was performed according to our previous method [22]. In brief, a polyethylene-10 (OD, 0.61 mm; ID, 0.28 mm) catheter was inserted into the rat's subarachnoid space through the L5–L6 intervertebral space, and the tip of the catheter was located at the L4 to L5 spinal segmental level. The specific PKR inhibitor 2-aminopurine (2-AP, Sigma) was dissolved in sterile normal saline containing 10% DMSO. The i.t. injections of the drug were performed 30 min before surgery and once daily 30 min before behavioral tests after SNL. The doses of 2-AP (0.25, 0.5 μmol/10 μl) used in the current experiment were based on previous studies [12, 23].

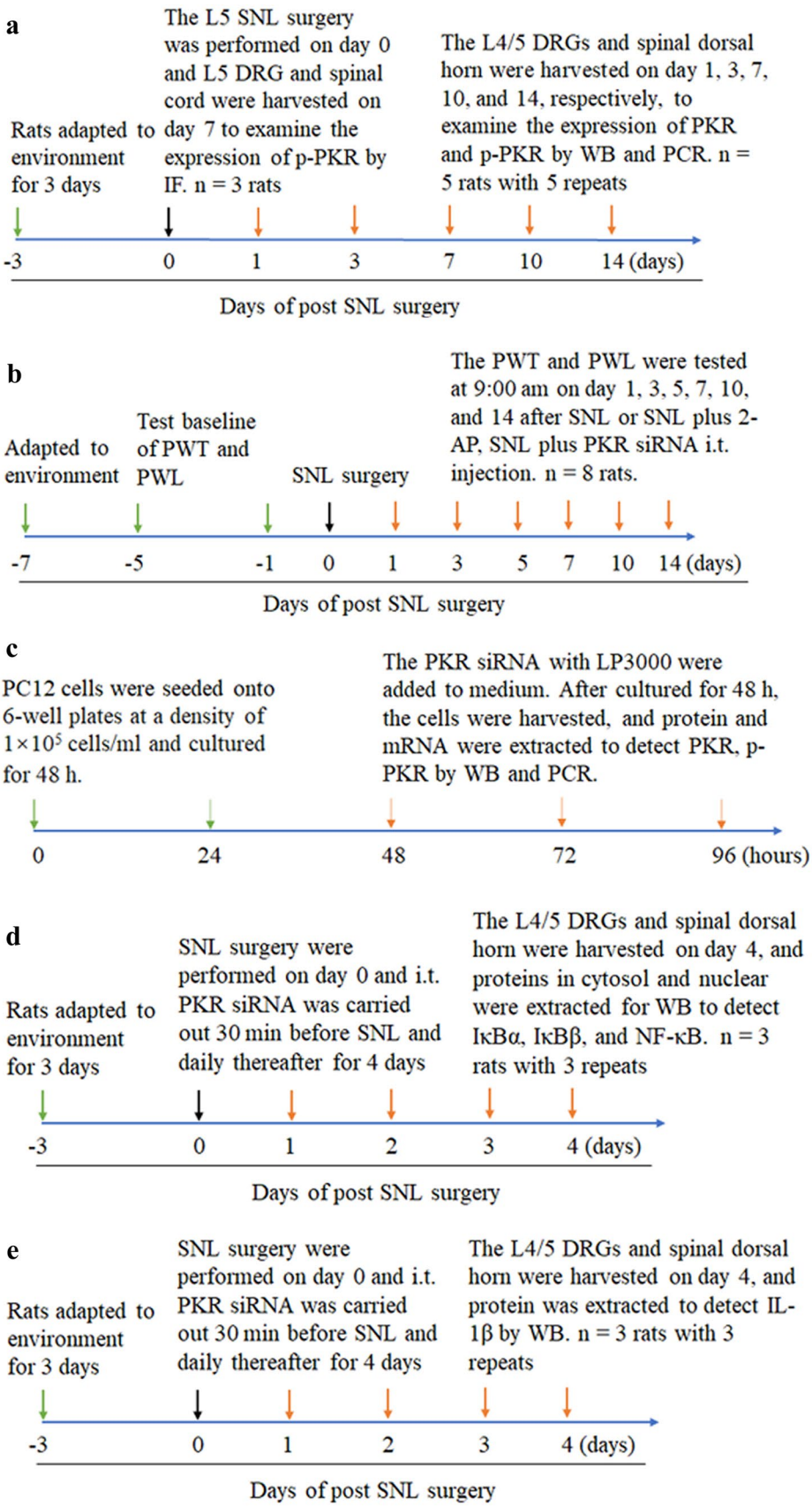


Fig. 1 Schematic illustration of the experiment design. **a** Expression of PKR mRNA, protein, and p-PKR in the DRG and spinal dorsal horn following SNL. **b** Effect of PKR activation in the DRG and spinal dorsal horn on the development of neuropathic pain. **c** PKR siRNA preparation and screening. **d** Effect of i.t. injection of PKR siRNA on the activation of NF-κB signaling in the DRG and spinal dorsal horn. **e** Effect of i.t. injection of PKR siRNA on the expression of IL-1β in the DRG and spinal dorsal horn

Behavioral Tests

The behavioral tests were performed following our previously described methods [24, 25]. All rats were adapted to the testing environment for at least 3 days before baseline measurement. The paw withdrawal threshold (PWT) to assess mechanical sensitivity was determined by applying von Frey hairs to the plantar surface of the hind paw, and 50% PWT was determined using the up–down method [26]. Heat hypersensitivity was evaluated by testing paw withdrawal latency (PWL) using a plantar analgesia tester (7370, Ugo Basile, Comeria, Italy) according to the method described by Hargreaves et al. [27]. A radiant heat source under the glass floor was aimed at the plantar surface of the hind paw. Three latency measurements were taken for each hind paw in each test session. The hind paws were tested alternately and the intervals between consecutive tests were more than 5 min. The three latency measurements for the right paw and the left paw were averaged separately. The entire behavioral tests were carried out blindly to the performer who did not know the experiment design.

Immunohistochemistry

Immunohistochemistry was done following our previous methods [20, 28]. Briefly, after defined survival times, control and nerve-injured rats were terminally anesthetized and perfused through the ascending aorta with normal saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the L4/5 DRGs and L4–5 spinal cord were removed and postfixed in the same fixative for 3 h, which was then replaced by 30% of sucrose-phosphate-buffered saline over two nights. Transverse DRG (16 μm) and spinal cord Sects. (25 μm) were cut on a cryostat (Leica, CM1950) and prepared for immunofluorescence staining. Sections were randomly selected and put into different wells of a 24-well plate. After washing with phosphate-buffered saline (PBS), the sections were blocked with 5% goat serum in 0.3% Triton X-100 for 1 h at 37 °C, and incubated with primary antibody overnight at 4 °C. For double immunofluorescence staining, the sections (except for IB4-treated DRG sections, which were only incubated with Cy3-conjugated secondary antibody) were incubated with a mixture of goat anti-mouse FITC-(1:200, Jackson ImmunoResearch, Amish, PA) and goat anti-rabbit Cy3–conjugated secondary antibody (1:400, Jackson ImmunoResearch) for 2 h at 37 °C. The stained sections were mounted onto slides and examined with an Olympus BX53 (Olympus Optical, Tokyo, Japan) fluorescence microscope. However, all sections of double immunofluorescence staining were examined by a high-resolution laser confocal fluorescence microscope (Nikon A1R MP+, Japan) scanned under a 1 μm-thick section, and images were captured with a CCD spot camera. The primary antibodies used in the experiment were rabbit anti-PKR (1:200; Cell Signaling Technology (CST), Danvers, MA), rabbit anti-phospho-PKR at Thr^{446} (1:200, Trevigen, Gaithersburg, MD USA), and rabbit anti-phospho-NF-κB p65 (Ser536) (1:200, CST). The following cell-specific markers were used: neurofilament-200 (NF-200, a marker for myelinated A-fibers, 1:200, Chemicon, Billerica, MA), FITC-conjugated isolectin B4 (IB4, a marker for unmyelinated nonpeptidergic C-fibers, 20 μg/ml, Sigma, St. Louis, MO), monoclonal anti-calcitonin-gene-related peptide (CGRP, a marker for unmyelinated peptidergic C-fibers, 1:200, GeneTex), glial fibrillary acidic protein (GFAP, a marker for astrocytes, 1:200; Chemicon), monoclonal neuronal-specific nuclear protein (NeuN, a neuronal marker, 1:200; Chemicon), OX42 (CD11b, a microglial marker, 1:200; Chemicon), CD3 (a marker of T-lymphocytes, 1:200; BD Pharmingen), and ED1 (a marker of monocyte/macrophages, 1:200; Millipore). The specificity of the PKR and p-PKR antibodies was examined by i.t. injection of PKR siRNA. The results of the immunofluorescence staining showed a clear decrease in PKR immunoreactivity (Fig. 10i-l) and the Western blotting showed reductions in the production of PKR protein and the level of p-PKR (Fig. 10c and d) in the spinal dorsal horn after rats received PKR siRNA treatment. The computer-assisted imaging analysis system (ImageJ; NIH, USA) was used to quantify the intensity of immunofluorescence following our published method previously [25]. An intensity threshold was set at the background level, firstly, to determine the total number of cells in a section. And then the threshold intensity was set above the background level to identify structures with positive staining signals. In the DRG, 5–6 slides per animal were counted. An average percentage relative to the total number of neurons was obtained for each animal across the different slides, and then the mean ± SE across animals was determined. In the spinal cord, the percentage of positive area in the dorsal horn was measured; but the method is the same as in the DRG.

Western Blotting

Western blotting was performed according to our previously published procedures [24, 29]. Briefly, the animals were sacrificed by decapitation at a designed time point. The lumbar 4/5 DRGs and L4–5 spinal dorsal horn were harvested and placed temporarily in liquid nitrogen. Next, the samples were homogenized with ice-cold lysis buffer (10 mM Tris, 5 mM EGTA, 0.5% Triton X-100, 2 mM benzamidine, 0.1 mM PMSF, 40 mM leupeptin, 150 mM NaCl, 1% phosphatase inhibitor cocktail II and III). Then, the crude homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C; the pellet (P1) and supernatant (S1, total soluble extraction) were collected in different tubes. The S1 was further centrifugation at 20,000 × *g* for 20 min at

Table 1 Sequences (5′-3′) of primers used

Gene	Forward primer	Reverse primer
PKR	GAAATTGGCTCGCGTGGATTTGG	TGGCGTGATTGAGTTCTGCTAGT
GAPDH	CATGGCCTCCAAGGAGTAAGAA	CCCTCCTGTTGTTATGGGGTC

4 °C. The supernatant (S2) containing cytosolic fractions was collected. The P1 was resuspended in 50 µL of hypertonic lysis buffer and incubated with shaking for 20 min at 4 °C. Then, the samples were centrifuged at 13,000 × *g* for 5 min at 4 °C. The supernatant (S3) containing nuclear extracts was collected. After the protein concentrations of S1, S2, and S3 were measured, the samples were heated for 5 min at 99 °C, and 30–60 µg protein was loaded onto 10% SDS–polyacrylamide gels. The proteins were electrophoretically transferred onto PVDF membranes. The blotting membranes were blocked with 3% non-fat milk for 1 h and incubated overnight at 4 °C with primary antibodies. The following primary antibodies were used: rabbit anti-PKR (1:1000; CST), rabbit anti-p-PKR-Thr^{446} (1:500; Trevigen, Gaithersburg, MD USA); rabbit anti-IL-1β (1:500; Abcam); rabbit anti-IL-6 (1:1000; Invitrogen); rabbit anti-TNF-α (1:500; Abcam); rabbit anti-INF-γ (1:500; ABclonal); rabbit anti-NF-κB phospho-p65-Ser^{346} (1:1000; CST), rabbit Anti-IκBα (1:500; Abcam), rabbit Anti-IκBβ (1:500; Abcam), mouse anti-calnexin (1:500; Santa Cruz), rabbit anti-H3 (1:2000, Millipore), and mouse anti-β-actin (1:10,000; Sigma). The proteins were detected with horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibodies (BIORad, 1:3000), visualized using the chemiluminescence reagents provided with the ECL kit (BIORad), and detected by a machine of ProteinSimple (FluorChem E, USA). The intensities of the blots were quantified by a computer-assisted imaging analysis system (ImageJ; NIH, USA). The ratios of a target protein to β-actin or calnexin and H3 were calculated for each animal. After the mean across sham animals was obtained, the ratio of each sham animal was divided by that of the mean. Then, the mean ± SE across sham animals was determined. Next, each ratio of SNL or drug-treated rats was divided by the mean of the control (sham) group. And then the mean ± SE, which represents the relative expression of SNL or drug-treated group to control (sham), across animals was determined.

RNA Extraction and Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR was performed following the methods described previously [20, 30]. After the rats were sacrificed by decapitation at a designed time point, the L4/5 DRGs and spinal dorsal horn were harvested for quantitative real-time RT-PCR. Total RNA was extracted via the Trizol method (Invitrogen/ThermoFisher Scientific). Reverse transcription was performed using oligo-dT primers and PrimeScript II RTase (TAKARA) according to the manufacturer's protocol. Each sample was run in triplicate in a 20 µl reaction volume which contains 10 µM each of forward and reverse primers, 10 µl of SYBR Green qPCR SuperMix (Invitrogen), and 25 ng of cDNA. Reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System. GAPDH was used as an internal control for normalization. The relative expression of PKR to GAPDH mRNA in the DRG and spinal cord were quantified by the $2-^{\Delta\Delta CT}$ method. The rat-specific primer sequences used to detect PKR and GAPDH mRNA in the qPCR analysis are listed in Table 1.

siRNA Preparation and Screening

Specific siRNAs were selected to knock down the expression of PKR in the spinal cord and DRG. Three siRNAs targeting rat PKR gene were designed and synthesized by GenePharma (Shanghai, China). These sequences were blast searched on the NCBI website to verify the specificity for PKR before manufacturing by GenePharma Inc. As a control, a scrambled non-targeting oligo designed by GenePharma was used. The nucleotide sequences of PKR siRNA are listed in Table 2. The PC12 cells were used to screen the effective PKR siRNA. PC12 cells (EK-Bioscience, Shanghai, RRID: CC-Y3028) were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco), which contained 10% fetal bovine serum (FBS, Gibco), and 1% antibiotics (Gibco). The cells were seeded at 1×10^5 cells per well in

Table 2 Sequences (5′-3′) of siRNA and scRNA

Gene	Forward	Reverse
siRNA 350	CCAAAUUAGCUGUCGAAAUTT	AUUUCGACAGCUAAUUUGGTT
siRNA 799	CCAUCUGAUGACGUGCUAATT	UUAGCACGUCAUCAGAUGGTT
siRNA 1455	GGGCCUUAUUCUAGCUGAATT	UUCAGCUAGAAUAAGGCCCTT
scRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

a 6-well plate and were incubated in a humidified incubator (Thermo, USA) with 5% CO_2 at 37 ℃. After 48 h, the siRNAs were transfected into the PC12 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) and cultured for another 48 h. The expression level of PKR was examined with qPCR and Western blotting. The expression of PKR mRNA was suppressed by 30.2%, 50.3%, and 41.9%, which were treated with PKR siRNA sequences 350, 799, and 1450, respectively. When the siRNA was delivered by i.t. injection, the TurboFect in vivo transfection reagent (Thermo Scientific Inc.) was used as a delivery vehicle to prevent degradation and enhance the cell membrane penetration of the siRNA. Our in vivo experiments showed that i.t. injection of PKR siRNA 350 remarkably suppressed the production of PKR protein in the dorsal horn. Hence, the synthesized PKR siRNA 350 was chosen for the subsequent experiments.

Statistical Analysis

Statistical tests were performed with SPSS 21.0 (SPSS Inc., USA). All data were presented as mean ± SE. For behavioral data, a two-way analysis of variance (ANOVA) with repeated measures followed by Tukey's post hoc test was used for difference over time. The one-way ANOVA followed by individual post hoc comparisons (Tukey's post hoc tests) were carried out for the data between groups at the same time points. For Western blot, qPCR, and immunohistochemistry data, the differences were tested using one-way ANOVA followed by individual post hoc comparisons (Tukey's post hoc tests) or using Student's *t*-test if only two groups were applied. A value of $P < 0.05$ was considered significant.

Results

Lumbar 5 Spinal Nerve Ligation (SNL) Resulted in Mechanical Allodynia, Thermal Hyperalgesia, and the Activation of PKR in the Ipsilateral DRGs

As reported previously, the L5 SNL in the current study resulted in reductions in the paw withdrawal threshold (PWT) (Fig. 2a) and paw withdrawal latency (PWL) (Fig. 2b) following surgery. Results of Western blotting showed a significant increase in the level of phosphorylated PKR (p-PKR) (Fig. 2c) in the L4/5 DRGs, which occurred on day 3 and peaked on day 7, after SNL. But the total PKR

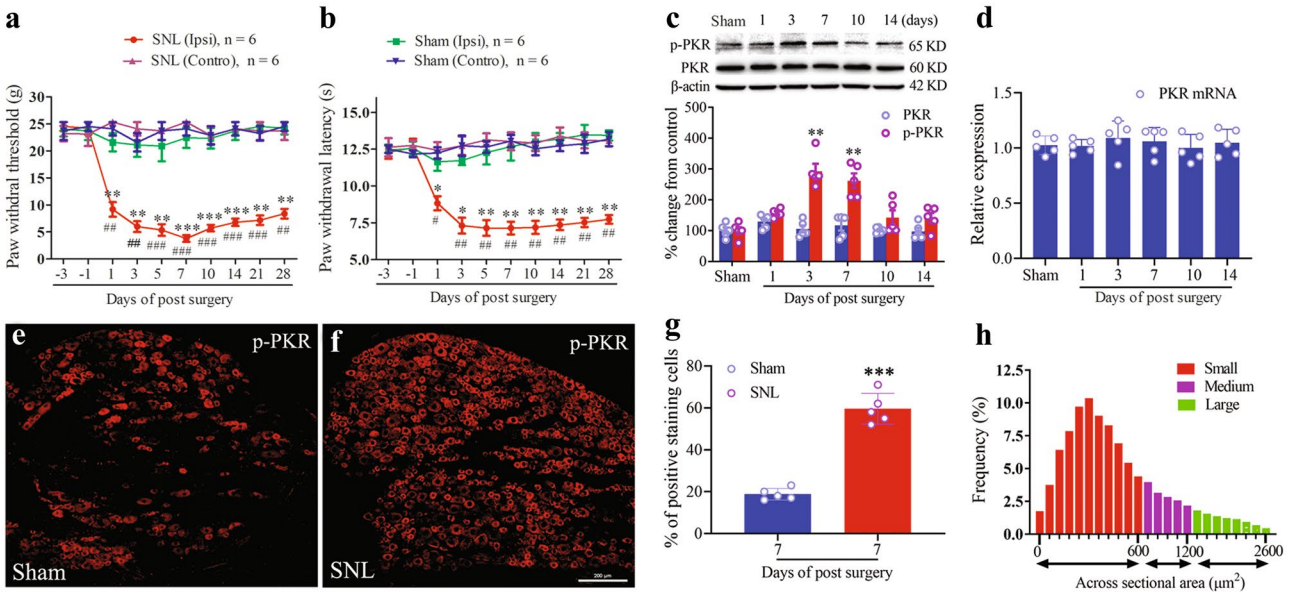


Fig. 2 Lumbar 5 spinal nerve ligation (SNL) resulted in abnormal pain and led to an increase in the level of phosphorylated PKR (p-PKR) in the DRG. **a**, **b** SNL led to significant reductions of mechanical paw withdrawal threshold (PWT) (**a**) and thermal paw withdrawal latency (PWL) (**b**) in the ipsilateral hind paw. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. sham group, one-way ANOVA. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. baseline (one day before SNL), two-way ANOVA. **c** Results of Western blotting showed a significant increase in the level of p-PKR, but not total PKR (t-PKR) protein, in the ipsilateral L4/5 DRGs following SNL. $^{**}P < 0.01$ vs. sham group, one-way ANOVA. **d** Real-time quantitative PCR (qPCR) assay showed there was no significant change of PKR mRNA in the ipsilateral L4/5 DRGs after SNL. **e**, **f** Representative images of immunofluorescence staining showing clear increased p-PKR positive staining cells in the DRG in SNL (**f**), but not sham (**e**), rats. Scale bar: (**e**, **f**) = 200 μm. **g** Quantitative analysis revealed a significant increase in p-PKR positive staining cells in the DRG 7 days after SNL. $^{***}P < 0.001$ vs. sham group, Student's *t*-test. **h** Histograms showing the frequency of p-PKR-labeled somata by cross-sectional area in the DRG in the SNL group

(t-PKR) protein (Fig. 2c) was not changed by the surgery. A quantitative RT-PCR (qPCR) assay showed that the PKR mRNA (Fig. 2d) was not changed by SNL. Results of immunofluorescence staining showed a remarkable increased p-PKR positive staining cells in the ipsilateral L5 DRG on day 5 after SNL (Fig. 2e–g). Histograms assay showed that the p-PKR positive staining cells were mainly distributed in the neurons across section area less than 600 μm^2 (about 74.01%), and a minimum distributed in the neurons across section area between 600–1200 μm^2 (about 14.71%) and 1200–2600 μm^2 (about 9.29%) (Fig. 2h).

To determine the cell type of PKR-expressing cells in the DRG, double immunofluorescence staining was performed in the sham group, firstly. The results showed that PKR colocalized with neurofilament-200 (NF-200, a marker of myelinated A-fibers, Fig. 3a–c), calcitonin-gene-related peptide (CGRP, a marker for unmyelinated peptidergic C-fibers, Fig. 3d–f), isolectin B4 (IB4, a marker of unmyelinated nonpeptidergic C-fibers, Fig. 3g–i), and glial fibrillary acidic protein (GFAP, a marker of satellite glial cells, Fig. 3j–l). To further determine and compare the cell type of p-PKR-expressing cells in the DRG in SNL group, the double immunofluorescence staining was also performed on day 5 after surgery. The results showed that p-PKR colocalized with NF-200 (Fig. 4a–c), CGRP (Fig. 4d–f), and IB4 (Fig. 4g–i), but not with GFAP (Fig. 4j–l).

The expression of p-PKR in T-lymphocytes and monocyte/macrophages in the DRG was also examined after SNL by double immunofluorescence staining. The results showed that p-PKR did not colocalize with CD3 (a marker of T-lymphocytes, Fig. 5a–c) and ED1 (a marker of monocyte/macrophages, Fig. 5d–f).

SNL Led to Increase in the Level of Phosphorylated PKR (p-PKR) in the Ipsilateral Spinal Dorsal Horn and Anterior Cingulate Cortex (ACC)

It has been demonstrated that the spinal dorsal horn, location of second order neurons relays pain message, plays critical role in genesis of peripheral neuropathic pain. In the current study, the expression and activation of PKR in the dorsal horn were examined following SNL. Results of Western blotting showed a significant increase in the level of p-PKR in the ipsilateral dorsal horn, which occurred on day 3, reached peak on day 7, and persisted to day 10 after SNL (Fig. 6a). However, the SNL did not lead to the changes of t-PKR protein (Fig. 6a) and mRNA (Fig. 6b) in the ipsilateral dorsal horn. Compared to the sham group, the level of p-PKR and t-PKR in the contralateral dorsal horn was not changed significantly by the SNL (Fig. 6c). Interestingly, the SNL also resulted in a significant increase in the level of p-PKR, but not PKR, in ipsilateral ACC, which occurred on day 7 and persisted to day 10 after surgery (Fig. 6d). Images of immunofluorescence staining exhibited that SNL led to a clear increase in the expression of p-PKR in the dorsal horn compared with that of sham rats (Fig. 6e, f). A quantitative assay of the p-PKR positive staining area in the dorsal horn showed a significant increase in the rats receiving SNL (Fig. 6g).

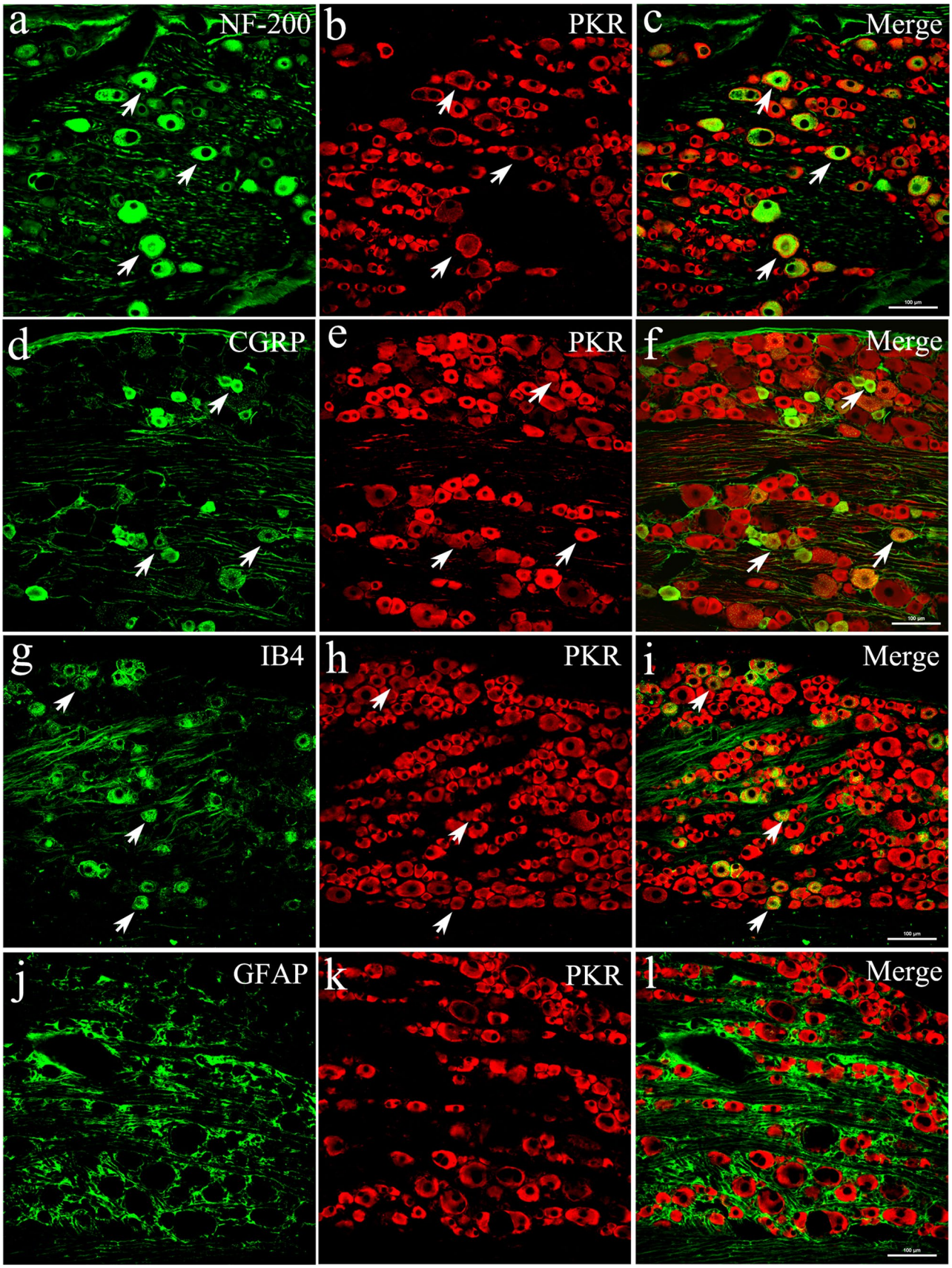
To observe the cell type of PKR-expressing cells in the dorsal horn, double-labeled immunofluorescence staining was carried out between PKR and NeuN (a marker of neuronal cells), GFAP (a marker of astrocytes), and OX-42 (a marker of microglia), respectively, in the sham group, firstly. The results showed that the PKR colocalized with NeuN (Fig. 7a–c), GFAP (Fig. 7d–f), and OX-42 (Fig. 7g–i). To further determine and compare the cell type of p-PKR-expressing cells in the SNL group, the expression of p-PKR in the dorsal horn was also examined on day 5 after surgery. The results showed that the p-PKR overlapped with NeuN (Fig. 8a–c), GFAP (Fig. 8d–f), and OX-42 (Fig. 8g–i).

Intrathecal (i.t.) Injection of 2-AP, a Specific Inhibitor of PKR Activation, Alleviated Mechanical Allodynia, and Thermal Hyperalgesia Following SNL

In view of the potential role of PKR activation in the pathogenesis of neuropathic pain, a procedure of repeat i.t. injections of 2-AP were carried out, which started half hour before SNL, and once daily thereafter for 4 days after surgery. Data from pain-related behavioral tests showed that the reductions of SNL-induced PWT (Fig. 9a) and PWL (Fig. 9b) were dose-dependently prevented by the treatment of 2-AP. To observe the effect of 2-AP treatment on the established neuropathic pain, the i.t. injection of 2-AP was performed on day 7 after SNL, a time point at which the neuropathic pain has been established fully, in another experiment. The treatment was carried out once daily thereafter and persisted to day 9 after SNL. Results of behavioral data showed the SNL-induced decrease in PWT (Fig. 9c) and PWL (Fig. 9d) was partially reversed by the treatment of repeat i.t. injections of 2-AP. To verify the effect of i.t. injection of 2-AP on the activation of PKR, the level of p-PKR and the production of PKR in the spinal dorsal horn and DRG were examined by Western blotting after the treatment. The results showed that SNL-induced increase in the level of p-PKR in the DRG (Fig. 9e) and dorsal horn (Fig. 9f) was inhibited by the treatment prior to i.t. injection of 2-AP.

Intrathecal Injection of PKR siRNA Impaired the Development and Maintenance of Neuropathic Pain Following SNL

To further verify the above results, a procedure of i.t. injection of PKR siRNA was carried out. Three PKR siRNAs (350, 799, and 1450) were provided by GenePharma


a
NF-200
b
PKR
c
Merge
d
CGRP
e
PKR
f
Merge
g
IB4
h
PKR
i
Merge
j
GFAP
k
PKR
l
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◂**Fig. 3** The cell type of PKR-expressing cells in the DRG in the sham group. **a–l** Double immunofluorescence staining in the ipsilateral L5 DRG between PKR (red; **b**, **e**, **h**, and **k**) and NF-200, a marker of myelinated A-fibers (green; **a**); CGRP, a marker of unmyelinated peptidergic C-fibers (green; **d**); IB4, a marker of unmyelinated non-peptidergic C-fibers (green; **g**); and GFAP, a marker of satellite glial cells (green; **j**), was performed. Two images were merged in **c**, **f**, **i**, and **l**. The results indicate colocalization of PKR with NF-200 (**c**), CGRP (**f**), IB4 (**i**), and GFAP (**l**) on day 7 after SNL. Scale bar: (**a–l**) = 100 μm

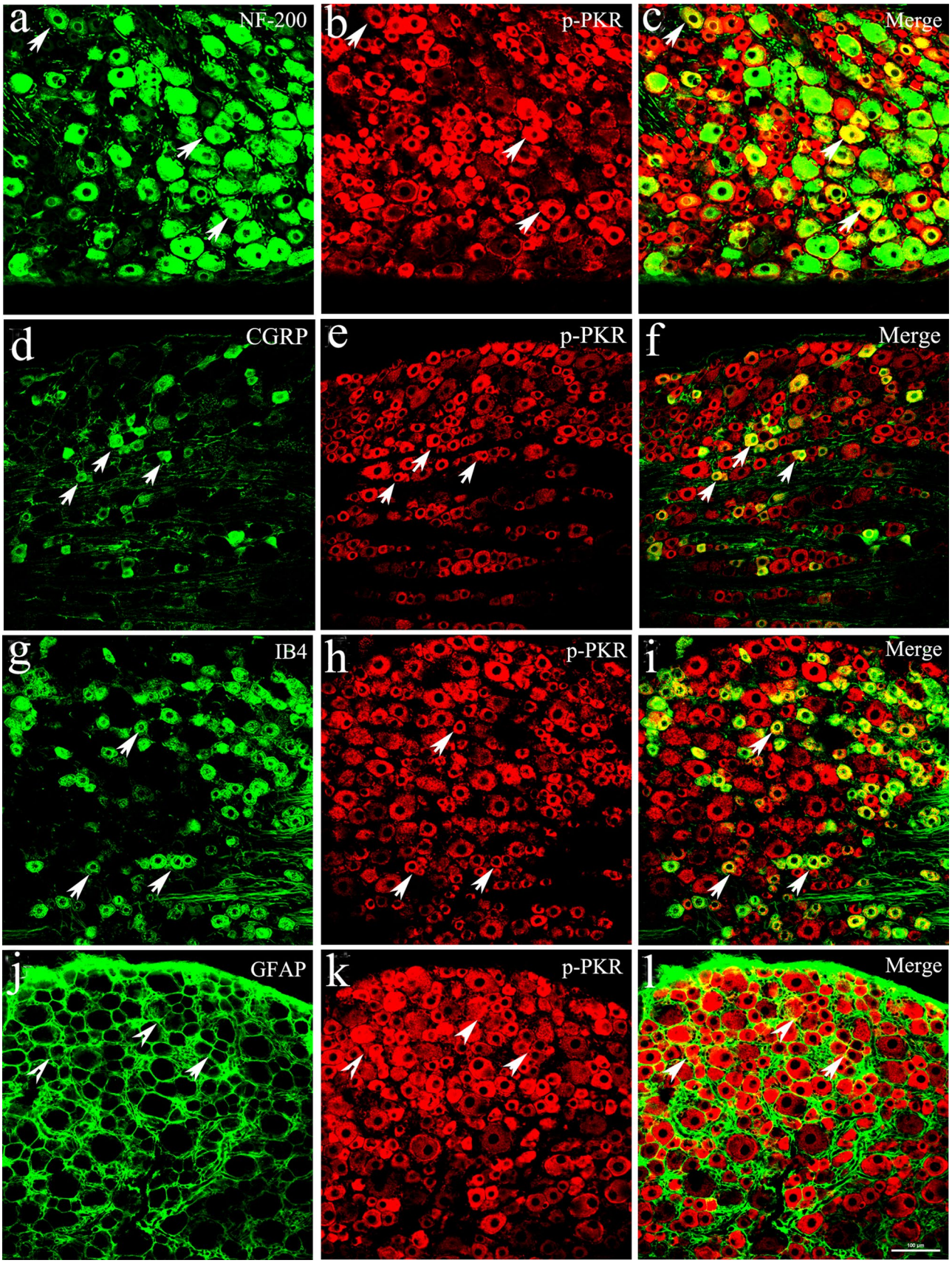
(Shanghai, China). To screen the effective one, these siRNAs were transfected to PC12 cells, and then the qPCR and Western blotting were performed to examine the expression of PKR in transfected PC12 cells. The results showed that the expression of PKR mRNA (Fig. 10a) and the production of PKR protein (Fig. 10b) were significantly inhibited by PKR siRNA350. To observe the in vivo effect, the repeat i.t. injections of PKR siRNA350 were performed 30 min before SNL and once daily thereafter for 3 days. Results of Western blotting showed that the production of PKR in the spinal dorsal horn (Fig. 10c) and DRG (Fig. 10d) were suppressed significantly by the treatment. The SNL-induced increase in the level of p-PKR in the dorsal horn and DRG was also inhibited by the treatment (Fig. 10c, d). Therefore, the PKR siRNA350 was used in the following studies. Results of behavioral tests showed that prior repeat i.t. injections of PKR siRNA, but not scRNA, resulted in a significant increase of PWT (Fig. 10e) and PWL (Fig. 10f) following SNL. The established mechanical allodynia (Fig. 10g) and thermal hyperalgesia (Fig. 10h) were also alleviated by the treatment of i.t. injection of PKR siRNA, which started on day 6 after SNL and once daily for 3 consecutive days. Results of immunofluorescence staining showed a clear decrease of PKR immunoreactivity in the DRG (Fig. 10i, j) and dorsal horn (Fig. 10k, l) in the rats treated with PKR siRNA (Fig. 10j, l), but not treated by scramble (sc) RNA (Fig. 10i, k), following SNL.

Prior i.t. Injection of PKR siRNA Prevented IκB Degradation and Reduced Nuclear Translocation of NF-κB p65 from Cytoplasm Following SNL

It has been reported that PKR activation leads to the phosphorylation and degradation of IκB, and thus promotes the nuclear translocation of NF-κB p65 [31]. In the current study, the changes of IκBα and IκBβ, two major forms of IkB, in the DRG and spinal dorsal horn were examined following SNL and SNL plus repeat i.t. injections of PKR siRNA. Results of Western blotting showed that SNL plus i.t. injection of vehicle (transfection reagent, TR) led to a significant decrease of IκBα and IκBβ in the DRG. But these decreases were reversed by the treatment prior to repeat i.t. injections of PKR siRNA (Fig. 11a). Moreover, the SNL resulted in a significant reduction of NF-κB p65 in the cytosol (Fig. 11b), and an increase of NF-κB p65 in the nucleus (Fig. 11c) in the same tissue as above. However, these changes of NF-κB p65 in cytosol and nucleus were reversed by the treatment of i.t. injection of PKR siRNA (Fig. 11b, c). The changes of IκB and NF-κB p65 in the spinal dorsal horn were also examined following SNL and SNL plus i.t. injection of PKR siRNA. The results showed that SNL plus i.t. injection of vehicle (TR) led to significant reductions of IκBα and IκBβ. But these reductions were reversed by the treatment prior to repeat i.t. injections of PKR siRNA (Fig. 11d). The SNL also resulted in a decrease of NF-κB p65 in the cytosol (Fig. 11e), and an increase of NF-κB p65 in the nucleus (Fig. 11f) in the dorsal horn. But these changes of NF-κB p65 in cytosol and nucleus were reversed by the treatment of i.t. injection of PKR siRNA (Fig. 11e, f). When the time course of the changes of IκBα and IκBβ were examined, the results showed that the reductions of IκBα and IκBβ in the DRG (Fig. 11g) and spinal dorsal horn (Fig. 11h) occurred on day 3, reached peak on day 7, and persisted to day 10 after SNL. Immunofluorescence staining shows that SNL led to clear nuclear translocation of NF-κB p65 in the DRG (Fig. 11i–k) and dorsal horn (Fig. 11l–n).

The SNL-Induced Increase in the Production of Proinflammatory Cytokines in the DRG and Spinal Dorsal Horn Was Suppressed by the Treatment of Repeat i.t. Injections of PKR siRNA

The previous study has shown that PKR activation regulates the expression of proinflammatory cytokines in the nervous system [11]. In the current experiment, the role of PKR activation in the production of proinflammatory cytokines IL-1β, IL-6, and TNF-α in the DRG and spinal dorsal horn was examined following SNL. Results of Western blotting showed that the SNL led to a significant increase in the production of IL-1β, including premature (31 KD) and mature IL-1β (17 KD), in the L4/5 DRGs (Fig. 12a) and spinal dorsal horn (Fig. 12b) following SNL. The significant increase of IL-1β in the DRG occurred on day 3 and persisted to day 10 (Fig. 12a), but in the spinal dorsal horn, this increase occurred on day 1, peaked on day 7, and persisted to day 14 (Fig. 12b) after SNL. Prior repeat i.t. injections of PKR siRNA reduced the level of p-PKR and inhibited the production of IL-1β in the DRG after SNL (Fig. 12c). The increase in the production of IL-1β in the L4-6 dorsal horn was also inhibited by the treatment (Fig. 12d), which followed a decrease of p-PKR in the dorsal horn (Fig. 12d). To further verify the effect of PKR activation on the regulation of proinflammatory cytokines expression, the productions of IL-6, TNF-α, and INF-γ in the DRG and spinal dorsal horn were also examined following the treatment prior to repeat i.t. injections of PKR siRNA or scramble (sc) RNA in sham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◂**Fig. 4** The cell type of p-PKR-expressing cells in the DRG in SNL group. **a–l** Double immunofluorescence staining in the ipsilateral L5 DRG between p-PKR (red; **b**, **e**, **h**, and **k**) and NF-200, a marker of myelinated A-fibers (green; **a**); CGRP, a marker of unmyelinated peptidergic C-fibers (green; **d**); IB4, a marker of unmyelinated nonpeptidergic C-fibers (green; **g**); and GFAP, a marker of satellite glial cells (green; **j**), was performed. Two images were merged in **c**, **f**, **i**, and **l**. The results showing colocalization of p-PKR with NF-200 (**c**), CGRP (**f**), IB4 (**i**), and GFAP (**l**). Scale bar: (**a–l**) = 100 μm

and SNL groups. The results of Western blotting showed that SNL led to significantly increased expression of IL-6, TNF-α, and INF-γ in the DRG (Fig. 12e) and spinal dorsal horn (Fig. 12f). Except the INF-γ, these increases were inhibited by the treatment prior to repeat i.t. injections of PKR siRNA, but not i.t. PKR scRNA.

Discussion

PKR, a double-stranded RNA (dsRNA)–activated serine/threonine kinase, is an important component in inflammation and immune dysfunction that regulates the MAPKs, IRF3, NF-κB, apoptosis, and autophagy pathways [9]. Herein, the role of PKR activation in the DRG and spinal dorsal horn in the pathogenesis of neuropathic pain was investigated. The results revealed that SNL-induced activation of PKR enhanced IκB degradation and promoted the nuclear translocation of NF-κB p65, subsequently increasing the expression of IL-1β, IL-6, and TNF-α in the DRG and spinal dorsal horn and contributing to the development and maintenance of neuropathic pain.

Compelling evidence has demonstrated the critical role of neuroinflammation in the genesis of neuropathic pain. Following peripheral nerve injury, glial cells and infiltrated immune cells, such as T-lymphocytes and monocytes/macrophages, in the DRG and spinal dorsal horn were activated progressively. After activation, these cells secrete proinflammatory mediators, such as chemokines, cytokines, and neurotrophins, which contribute to the development and maintenance of neuropathic pain by regulating the cross talk between neurons and glial cells [14, 32–38]. PKR, a ubiquitously expressed serine/threonine kinase, has been implicated as a signal integrator in translational and transcriptional control pathways [8]. In addition to dsRNA and interferon (IFN), PKR has been shown to be activated by cytokines such as TNF-α and IL-3 and lipopolysaccharide (LPS). Active PKR mediates the activation of MAPKs, inhibitors of κB (IκB) kinase (IKK), and IFN-β–promoter simulator 1 (IPS-1) signaling and then affects various transcription factors, including IRF3 and NF-κB, which are required for the expression of genes encoding proinflammatory cytokines [9, 39, 40]. In the present study, we found that SNL significantly increased the level of p-PKR in both the DRG and dorsal horn. Activated PKR was distributed in not only neurons but also glial cells, such as satellite glial cells, astrocytes, and microglia, but not in T-lymphocytes and macrophages. Although the activated PKR exhibited a remarkable expression in the DRG and dorsal horn in the SNL group, the PKR protein and PKR mRNA were not increased in both the DRG and spinal dorsal horn following SNL. It suggests that the SNL only promotes PKR activation, but does not increase its expression. In addition, inhibition of

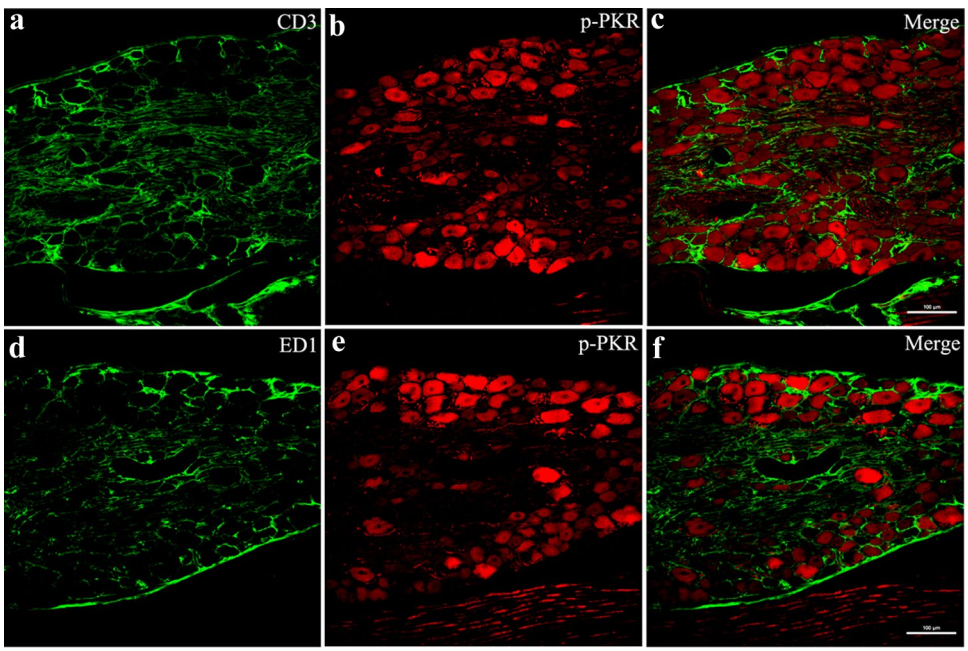

Fig. 5 Expression of p-PKR in T-lymphocytes and monocyte/macrophages in the DRG following SNL. **a–f** The representative images of double immunofluorescence staining between p-PKR (red; **b** and **e**) and CD3, a marker of T-lymphocytes (green; **a**), and ED1, a marker of monocyte/macrophages (green; **d**) showing no colocalization of p-PKR with CD3 (**c**) and ED1 (**f**). Scale bar: (**a–f**) = 100 μm

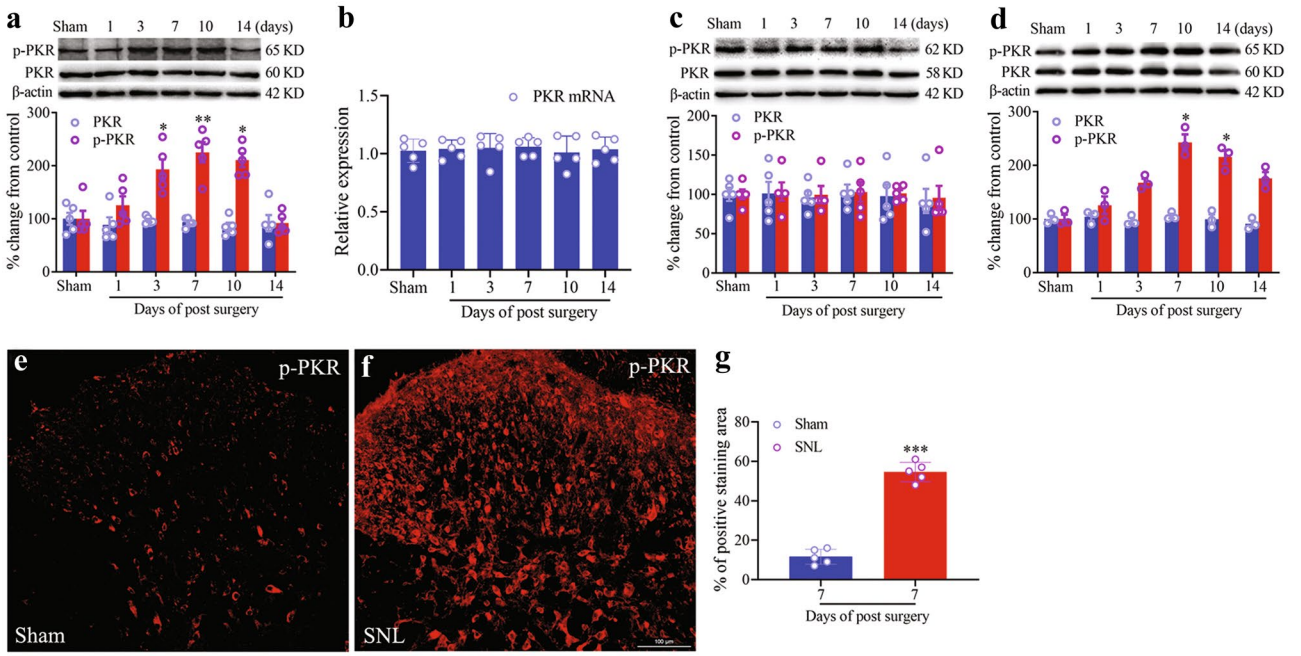

Fig. 6 Expression and activation of PKR in the spinal dorsal horn and anterior cingulate cortex (ACC) following SNL. **a** Results of Western blotting showed a significant increase in the level of p-PKR (**a**), but not total PKR protein (**a**), in the ipsilateral dorsal horn following SNL. $^{*}P<0.05$, $^{**}P<0.01$ vs. sham group, one-way ANOVA. **b** The PKR mRNA was not changed in the dorsal horn by the SNL. **c** The SNL did not lead to changes of p-PKR and total PKR protein in the contralateral dorsal horn. **d** The SNL resulted in a significant increase in the level of p-PKR, but not total PKR protein, in the contralateral ACC. $^{*}P<0.05$ vs. sham group, one-way ANOVA. **e**, **f** Results of immunofluorescence staining showing clear increased p-PKR positive staining cells in the ipsilateral dorsal horn in SNL (**f**), but not sham (**e**), group. Scale bar: (**e**, **f**) = 100 μm. **g** Quantitative analysis revealed a significant increase in p-PKR positive staining area in the dorsal horn 7 days after SNL. $^{***}P<0.001$ vs. sham group, Student's *t*-test

PKR by i.t. injection of 2-AP, a specific inhibitor of PKR activation, and i.t. injection of PKR siRNA prevented the development of neuropathic pain and impaired established neuropathic pain following SNL. A previous study demonstrated that intrathecal drug delivery can result in drug distribution to both the spinal cord and DRG [11]. In the current study, we found that the SNL-induced increase in the level of p-PKR in both the DRG and spinal dorsal horn was inhibited by i.t. injection of 2-AP and PKR siRNA. This finding indicates that PKR activation in the DRG and spinal dorsal horn is required for the pathogenesis of neuropathic pain. Our previous study [41] and others [42, 43] showed that peripheral nerve injury increases the production of TNF-α, which plays an important role in the genesis of neuropathic pain, in the DRG and dorsal horn. Moreover, TNF-α is a potent activator of PKR [44, 45]. Therefore, SNL-induced PKR activation in the current study may also result from the increased production of TNF-α in the DRG and spinal dorsal horn.

It has been well established that the NF-κB pathway–mediated expression of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, plays an important role in the development and maintenance of neuropathic pain following peripheral nerve injury [46–50]. NF-κB functions as a dimeric transcription factor composed of members of the Rel family, most often p50 and p65 (RelA). NF-κB is sequestered by an IκB complex (IκBα and IκBβ) in the cytosol in unstimulated cells. Phosphorylation of the IκB complex by IκB kinase (IKK) after cell surface receptor activation (e.g., TNFR and Toll-like receptors) triggers the proteasomal degradation of IκB and disruption of the cytosolic IκB-NF-κB complex, allowing NF-κB to translocate from the cytoplasm to the nucleus, where it binds to specific sequences of DNA and induces gene expression [51, 52]. Emerging evidence has shown that PKR activates IκB kinase (IKK), leading to the degradation of the inhibitors IκBα and IκBβ and the concomitant release of NF-κB, thus promoting the nuclear translocation of NF-κB and triggering the expression of genes encoding proinflammatory cytokines [31, 53]. In the current study, we found that repeated i.t. injections of PKR siRNA prevented SNL-induced nuclear translocation of NF-κB p65 by inhibiting

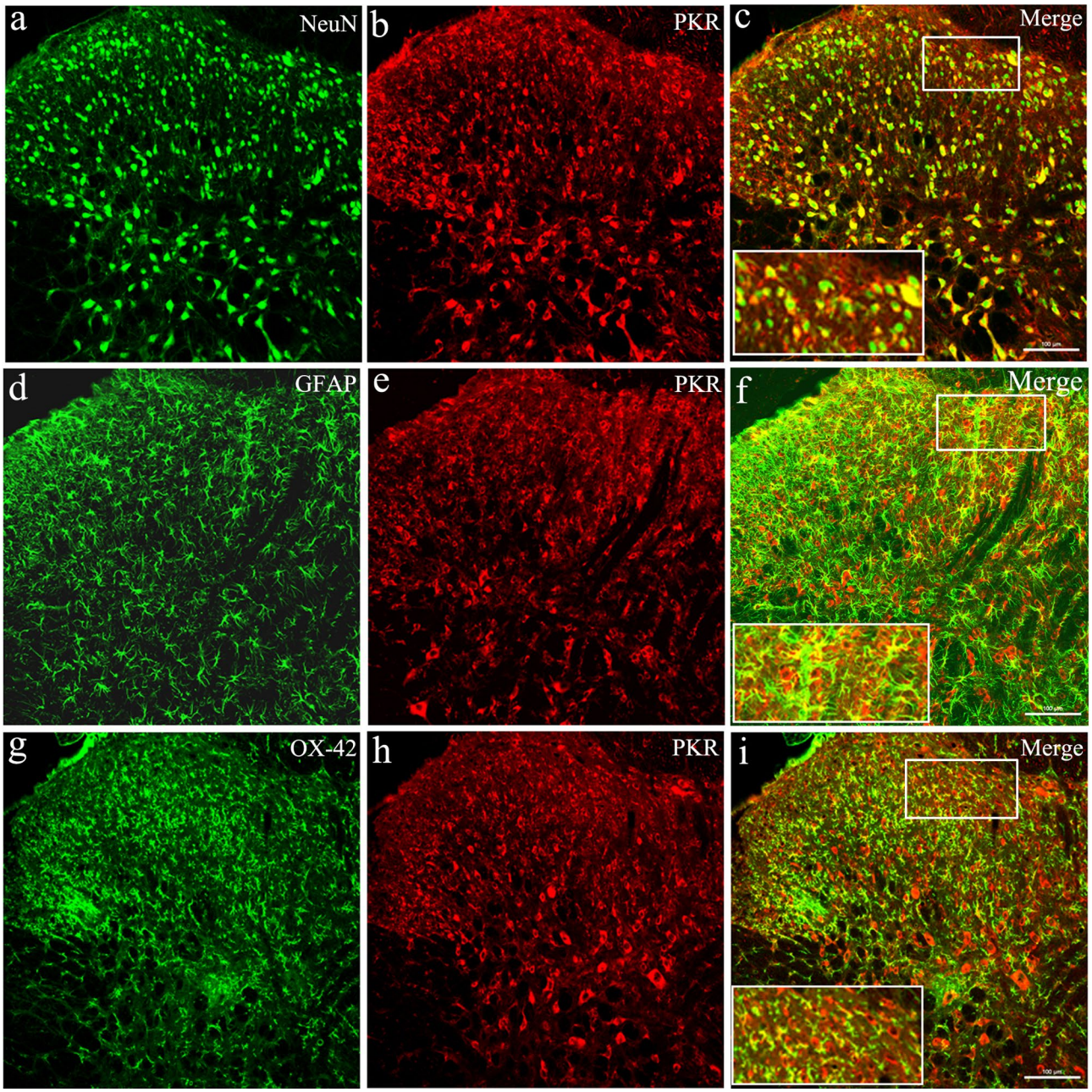

Fig. 7 The cell type of PKR-expressing cells in the spinal dorsal horn in the sham group. **a**–**i** Representative images showing the results of double immunofluorescence staining in the L5 ipsilateral dorsal horn between PKR (red; **b**, **e**, and **h**) and NeuN (green; **a**), GFAP (green; **d**), and OX-42 (green; **g**). The results indicate that PKR is in neurons (**c**), astrocytes (**f**), and microglia (**i**). Scale bar: (**a**–**i**) = 100 μm

the degradation of IκBα and IκBβ in the cytosol. Moreover, the SNL-induced increase in the production of IL-1β, IL-6, and TNF-α in the DRG and spinal dorsal horn was effectively inhibited by this treatment. This finding suggests that SNL-induced PKR activation activates the NF-κB pathway and mediates the increased expression of IL-1β, IL-6, and TNF-α in the DRG and spinal dorsal horn. A recent study has revealed that optogenetic activation of spinal microglia might be via elevating Ca^{2+}, promoting the increased release of IL-1β and mediating the development of chronic pain [54]. Therefore, whether the elevated-Ca^{2+} triggers the PKR activation in microglia and thus increases the release of IL-1β needs to be verified in the future. It has previously been reported that the increase in the production of

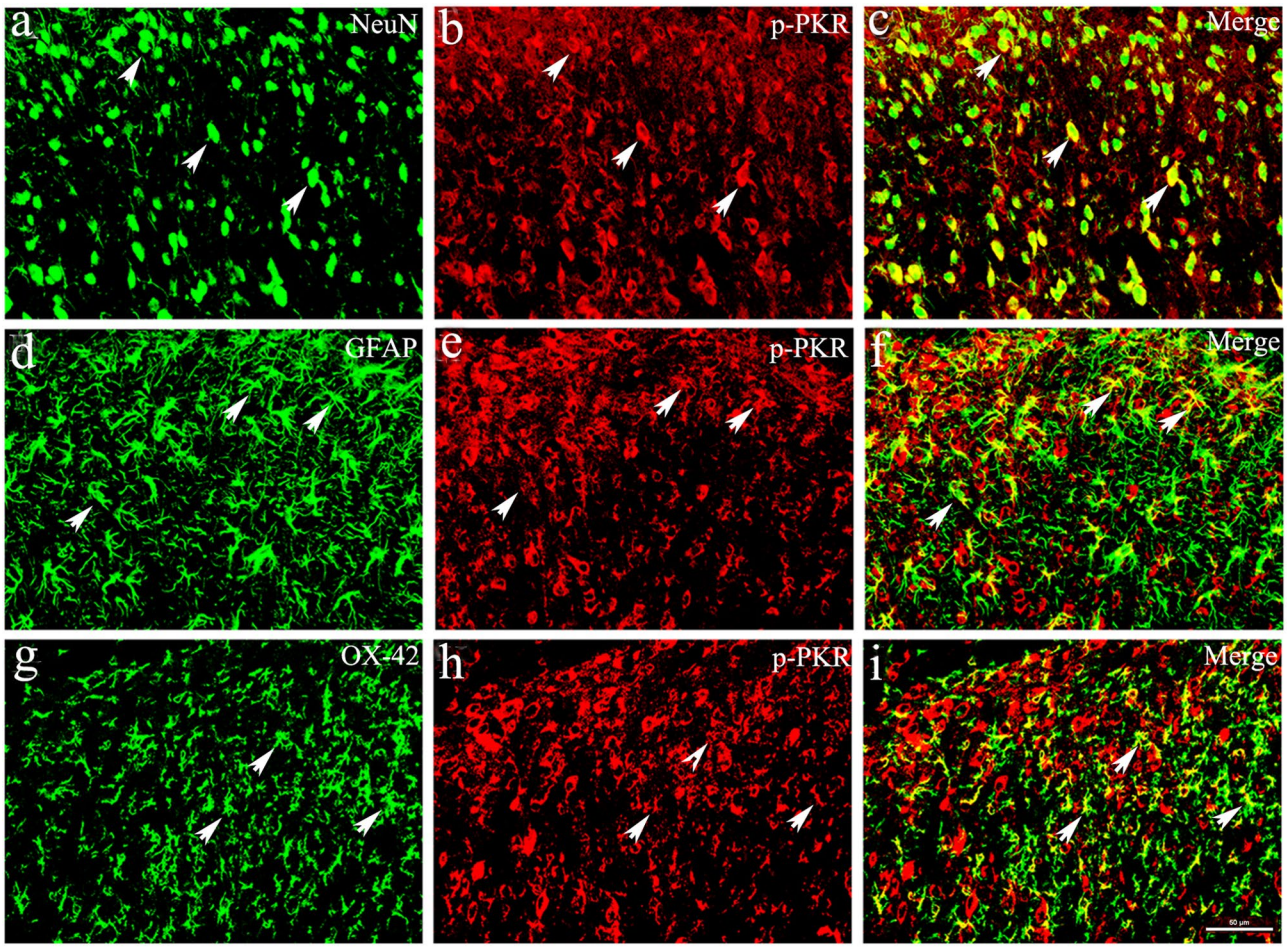

Fig. 8 The cell types expressing p-PKR in the spinal cord following SNL. **a**–**i** Representative images showing the results of double immunofluorescence staining in the L5 ipsilateral dorsal horn between p-PKR (red; **b**, **e**, and **h**) and NeuN, a neuronal marker (green; **a**); GFAP, an astrocyte marker (green; **d**); and OX-42, a microglia marker (green; **g**). The results indicate that p-PKR is in neurons (**c**), astrocytes (**f**), and microglia (**i**) 7 days after L5 SNL. Scale bar: (**a**–**i**) = 50 μm

interferon-gamma (INF-γ) in the DRG and spinal dorsal horn contributes to the genesis of chronic pain [55]. Moreover, INF-γ is a potent activator of PKR in the immune response [8, 44]. In the present study, we observed a significant increase in the production of INF-γ in the DRG and spinal dorsal horn following SNL. However, this increase was not inhibited by i.t. injection of PKR siRNA. This finding suggests that the regulation of INF-γ expression is not dependent on the activity of PKR. Compelling evidence has demonstrated that in addition to IKK, PKR also mediates the activation of MAPKs and IFN-β–promoter simulator 1 (IPS-1) signaling and contributes to the development of inflammatory diseases by affecting various transcription factors [9, 39, 40]. Therefore, whether SNL-induced PKR activation also leads to MAPKs and IPS-1 signaling activation, which have been shown to play critical roles in the pathogenesis of neuropathic pain, needs to be further studied. In addition, we have detected a significant increase in the level of p-PKR in ACC, a region which has been demonstrated to play key roles in relaying or governing pain-related messages [56–58]. Therefore, the role of PKR activation in the ACC in neuropathic pain also needs to be further studied. Moreover, it should be emphasized that the present study was carried out on male rats. Whether the PKR also acts in female rats still requires further study because previous studies have shown different reactions in male and female rats in the pathogenesis of chronic pain [59].

In summary, our current study demonstrated that peripheral nerve injury–induced PKR activation via NF-κB pathway activation in the DRG and spinal dorsal horn contributes to the pathogenesis of neuropathic pain. Targeting PKR might be a promising therapeutic strategy for the treatment of chronic pain.

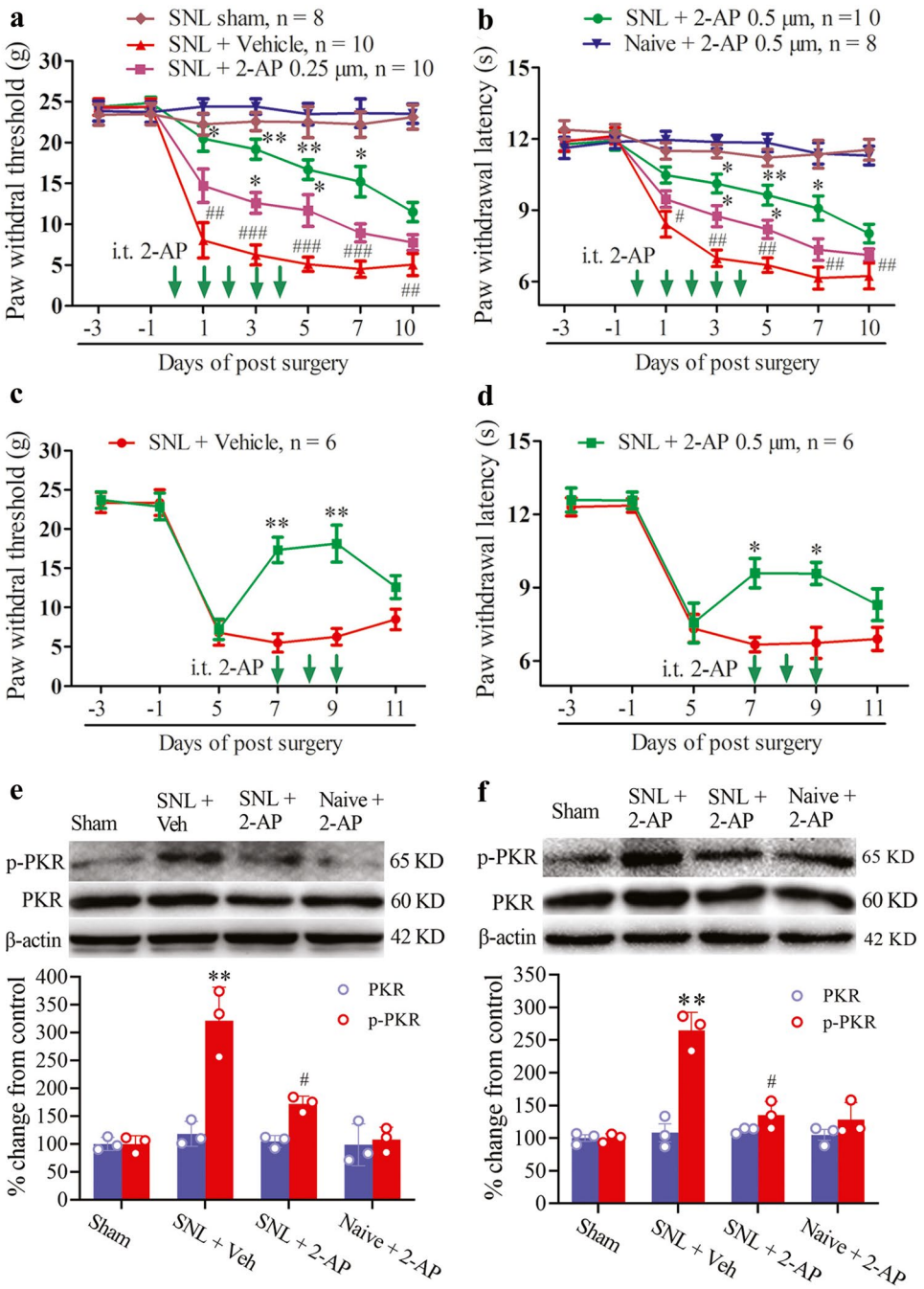

Fig. 9 Effect of intrathecal (i.t.) injection of 2-AP, a specific inhibitor of PKR activation, on the development and maintenance of neuropathic pain following SNL. **a**, **b** Results from behavioral tests showed that the reductions of paw withdrawal threshold (PWT) (**a**) and paw withdrawal latency (PWL) (**b**) following SNL were dose-dependently prevented by the treatment of i.t. injection of 2-AP. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. baseline (one day before SNL), two-way ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. SNL plus vehicle group, one-way ANOVA. **c**, **d** The established mechanical allodynia (**c**) and thermal hyperalgesia (**d**) were partially reversed in the group of SNL plus i.t. injection of 2-AP. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. SNL plus vehicle group, Student's *t*-test. **e**, **f** The SNL-induced increase in the level of p-PKR in the DRG (**e**) and dorsal horn (**f**) was inhibited by the treatment prior to i.t. injection of 2-AP. $^{**}P < 0.01$ vs. sham group; $^{\#}P < 0.05$ vs. SNL plus vehicle (Veh) group, one-way ANOVA

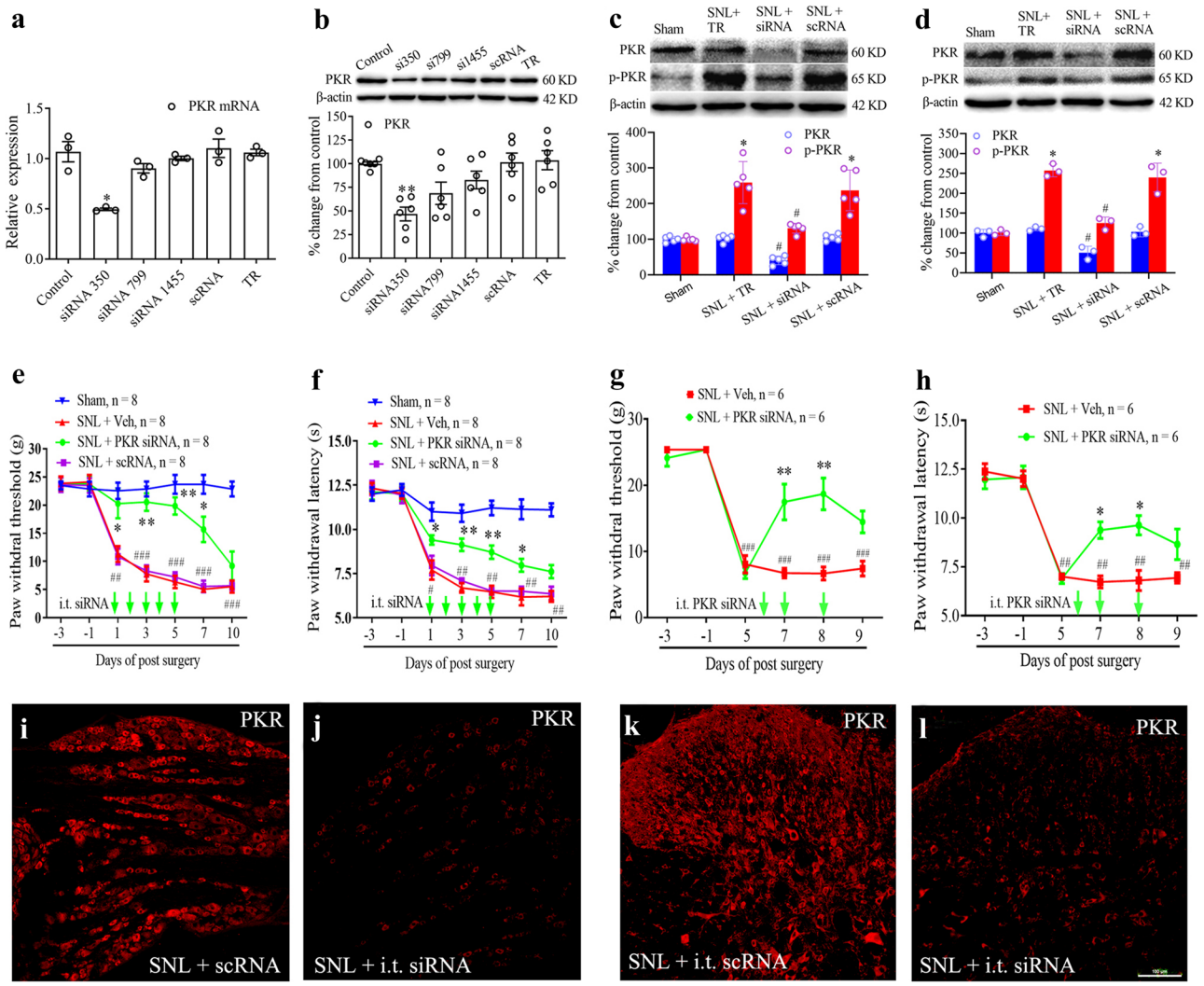

Fig. 10 Effect of i.t. injection of PKR siRNA on the development and maintenance of neuropathic pain following SNL. **a**, **b** Results from in vitro study showing that the expression of PKR mRNA (**a**) and protein (**b**) in cultured PC12 cells was inhibited by the treatment of PKR siRNA, especially siRNA350. $^{*}P<0.05$, $^{**}P<0.01$ vs. control (Con) or scramble (sc) RNA, one-way ANOVA. **c**, **d** Results of Western blotting assay showing that the production of PKR protein and SNL-induced increase in the level of p-PKR in the spinal dorsal horn (**c**) and L4/5 DRGs (**d**) was inhibited by the treatment of prior to repeat i.t. injections of PKR siRNA350. $^{*}P<0.05$ vs. sham group; $^{\#}P<0.05$ vs. SNL plus transfection regent (TR) group, one-way ANOVA. **e**, **f** Prior i.t. injection of PKR siRNA prevented the reductions of PWT (**e**) and PWL (**f**) following SNL. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. baseline (one day before SNL), two-way ANOVA; $^{*}P<0.05$, $^{**}P<0.01$ vs. SNL plus vehicle (Veh) group, one-way ANOVA. **g**, **h** The established mechanical allodynia (**g**) and thermal hyperalgesia (**h**) were partially reversed by the treatment of i.t. injection of PKR siRNA, which started on day 6 after SNL. $^{*}P<0.05$, $^{**}P<0.01$ vs. SNL plus vehicle (Veh, transfection regent) group, Student's *t*-test. **i–l** Results of immunofluorescence staining showing a significant reduction of PKR immunoreactivity in the DRG (**j**) and dorsal horn (**l**) in rats of SNL plus i.t. injection of PKR siRNA, but not SNL plus i.t. injection of PKR scRNA (**i**, **k**). Scale bar: (**i–l**) = 100 μm

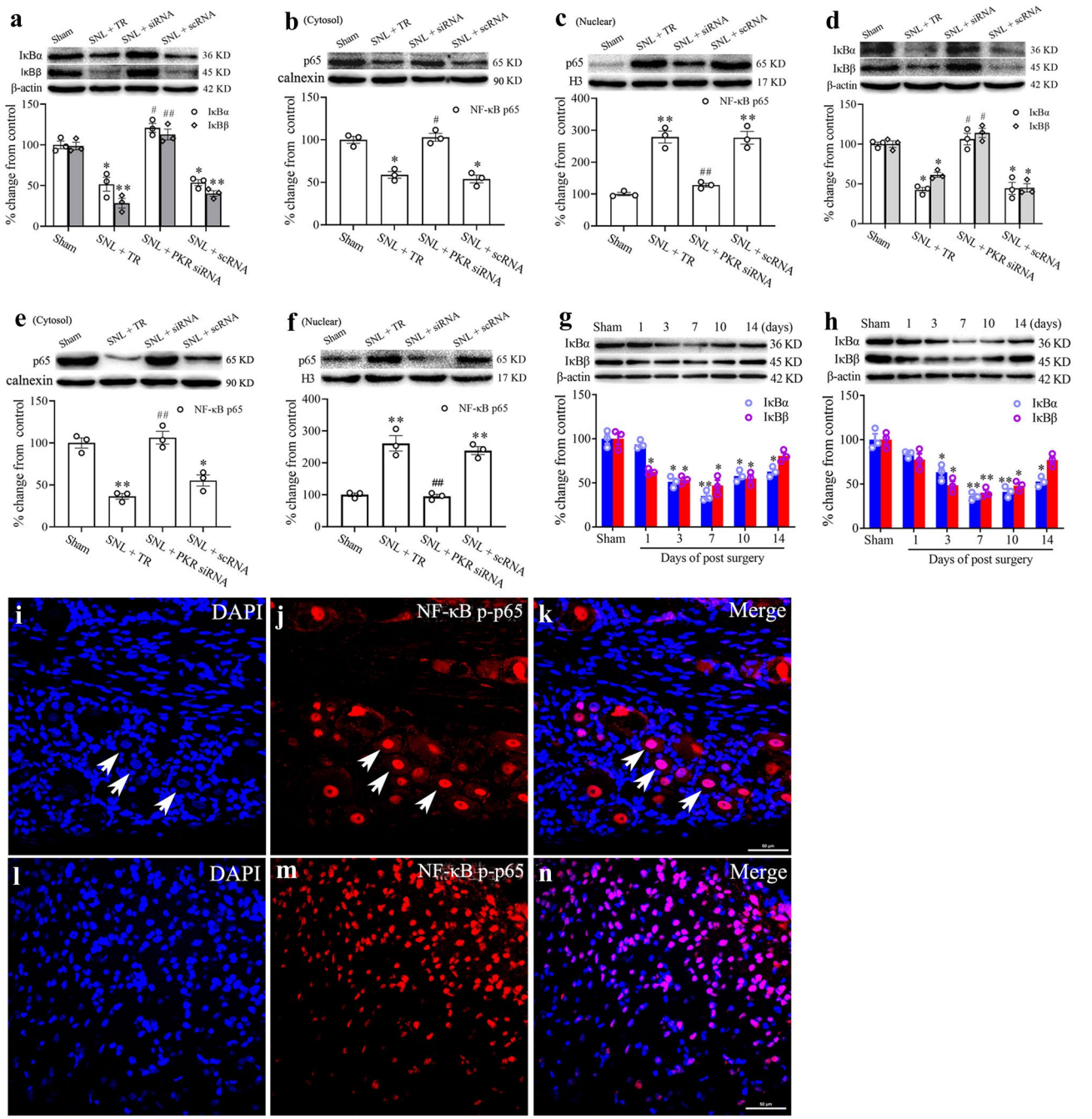

Fig. 11 Effect of PKR inhibition on NF-κB activation in the DRG and spinal dorsal horn following SNL. **a** The SNL-induced degradation of IκBα and IκBβ in the DRG was prevented by the treatment prior to repeat i.t. injections of PKR siRNA. **b**, **c** The reduction of NF-κB p65 in the cytosol (**b**) and the increase in the nucleus (**c**) in the DRG following SNL were reversed by the treatment prior to i.t. injection of PKR siRNA. **d** The SNL-induced degradation of IκBα and IκBβ in the dorsal horn was prevented by the treatment prior to repeat i.t. injections of PKR siRNA. **e**, **f** The reduction of NF-κB p65 in the cytosol (**e**) and the increase in the nucleus (**f**) in the dorsal horn following SNL were reversed by the treatment of prior to i.t. injection of PKR siRNA. $^{*}P<0.05$, $^{**}P<0.01$ vs. sham group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. SNL plus TR (transfection regent) group, one-way ANOVA (**a**–**f**). **g**, **h** The SNL led to a significant decrease of IκBα and IκBβ in the DRG (**g**) and spinal dorsal horn (**h**). $^{*}P<0.05$, $^{**}P<0.01$ vs. sham group, one-way ANOVA. **i**–**n** Results of immunofluorescence staining showing nuclear translocation of NF-κB p65 in the DRG (**i**–**k**) and spinal dorsal horn (**l**–**n**) in SNL rats. Scale bar: (**i**–**n**) = 50 μm

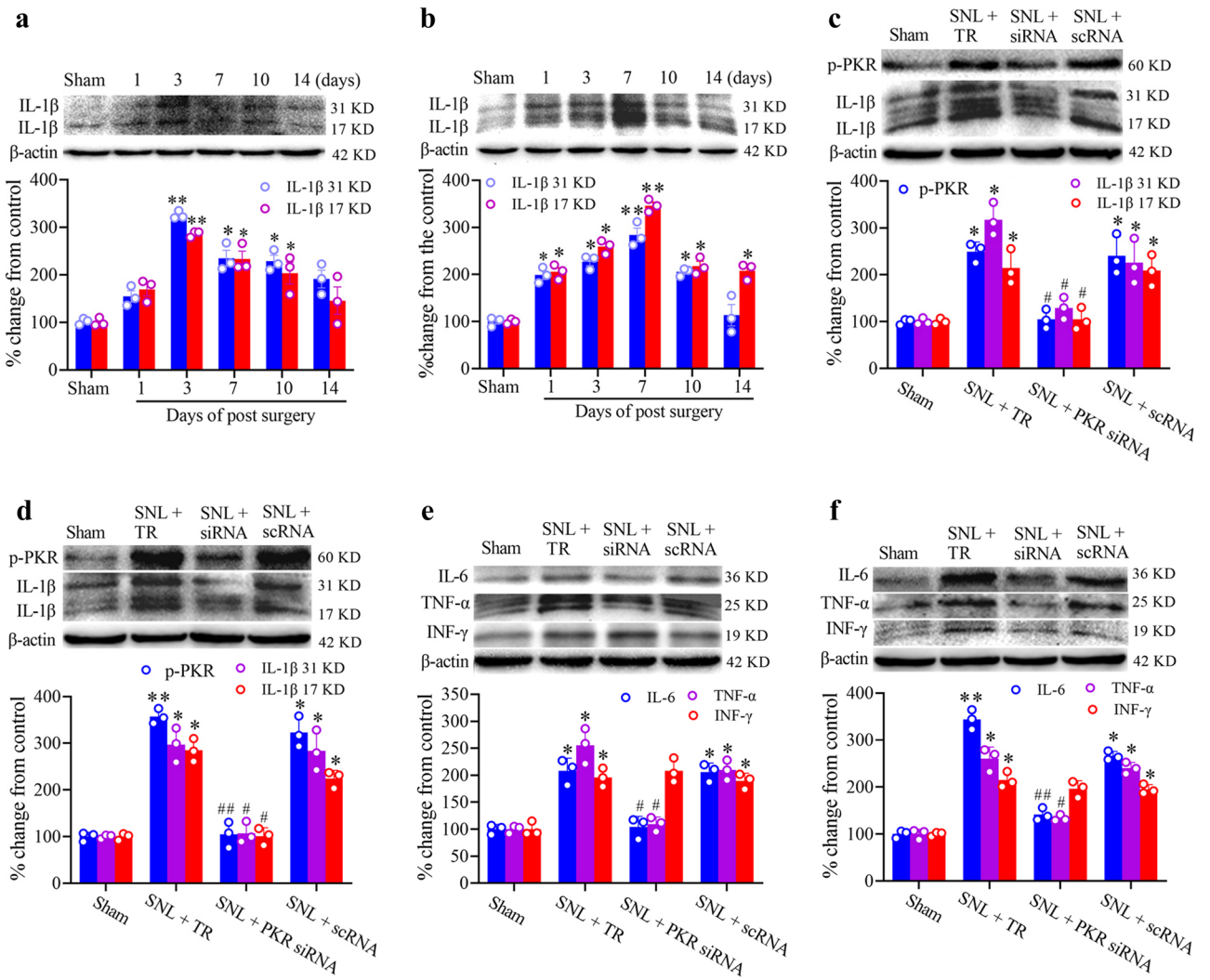

Fig. 12 Prior i.t. injection of PKR siRNA inhibited the production of IL-1β, IL-6, and TNF-α in the DRG and spinal dorsal horn following SNL. **a**, **b** SNL resulted in a significant increase in the production of IL-1β in the DRG (**a**) and spinal dorsal horn (**b**). $^{*}P<0.05$, $^{**}P<0.01$ vs. sham group, one-way ANOVA. **c**, **d** The SNL-induced increase in the production of IL-1β in the DRG (**c**) and spinal dorsal horn (**d**) was inhibited by the treatment prior to repeat i.t. injections of PKR siRNA. The increase in the level of p-PKR in the DRG (**c**) and spinal dorsal horn (**d**) was also reduced by the treatment. **e**, **f** SNL led to a remarkable increase in the production of IL-6, TNF-α, and INF-γ in the DRG (**e**) and spinal dorsal horn (**f**). Except INF-γ, the increase of IL-6 and TNF-α was repressed by the treatment prior to repeat i.t. injections of PKR siRNA. $^{*}P<0.05$, $^{**}P<0.01$ vs. sham group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. SNL plus TR (transfection regent) group, one-way ANOVA (**c**–**f**)

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Author Contribution JTX conceived the project and supervised all experiments. JTX, JZ, and XZ designed the project. JZ, XZ, and LB produced the animal model, conducted the behavioral experiments, and carried out the Western blot assays and siRNA transfection. YG, YY, and LL performed the PCR and immunohistochemistry experiments. LW, XW, and YQ carried out the cell culture. JZ, XZ, and JTX analyzed the data and wrote the manuscript. All the authors read and discussed the manuscript.

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Declarations

Ethics Approval All procedures used were approved by the Animal Care and Use Committee at Zhengzhou University, People's Republic of China.

Competing Interests The authors declare competing interests.

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