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CELLULAR AND MOLECULAR BIOLOGY

LncRNA KCNQ1OT1 promotes the apoptosis and inflammatory response of microglia by regulating the miR-589-5p/NPTN axis after spinal cord injury

ZHAOMING CHU, YOU LU, RUJIE QIN & YUEFU DONG

Abstract: Spinal cord injury (SCI) is a devastating traumatic condition accompanied with excessive inflammatory response and apoptosis of microglia. Long noncoding RNAs (lncRNAs) have been confirmed to be key regulators of cell inflammatory response. Nevertheless, the role of lncRNA KCNQ1OT1 in microglia apoptosis or inflammatory response after SCI remains to be explored. Our study focused on exploring the role and mechanism of KCNQ1OT1 in microglia after SCI. RT-qPCR showed that SCI induced the increase of KCNQ1OT1 level in mice spinal cord. Inhibition of KCNQ1OT1 suppressed the inflammatory response and apoptosis of microglia. In addition, KCNQ1OT1 was proved to bind with miR-589-5p, and NPTN was directly targeted by miR-589-5p. Furthermore, KCNQ1OT1 was negatively correlated with miR-589-5p and positively associated with NPTN. Rescue assays indicated that NPTN overexpression reversed the anti-inflammatory and anti-apoptosis effects of KCNQ1OT1 silencing. In summary, these data revealed that KCNQ1OT1 promoted inflammatory response and apoptosis of microglia by regulating the miR-589-5p/NPTN axis after SCI, which may offer a novel promising therapeutic target for SCI.

Key words: KCNQ1OT1, miR-589-5p, NPTN, spinal cord injury.

INTRODUCTION

Spinal cord injury (SCI), a devastating disabling condition, contributes to severe muscle weakness, movement dysfunction, and sensational changes (Ramer et al. 2014), which has received increasing attention from researchers all over the world (Cai et al. 2019). Every year, nearly 500,000 people suffer from SCI (Holmes 2017). There are many factors contributing to SCI including falls, road traffic accidents, inflammatory injuries, and spinal tumors (Hatch et al. 2017, Zhang et al. 2013). Although numerous medical technologies have been applied to improve the outcome of patient with SCI, it is still limited in the effective therapy for SCI (Thuret et al. 2006). Previous studies have revealed that the mechanism of SCI might be correlated with the primary damage resulting from initial mechanical injury and secondary damage; however, it is still largely unclear about the specific mechanism of SCI. Therefore, it is pressing to uncover more effective therapeutic methods of SCI.

Long noncoding RNAs (lncRNAs) are transcripts consisting of over 200 nucleotides without protein-coding potential (Wang et al. 2011). Although lacking protein-coding function, lncRNAs can also exert their function in various physiological processes (Fatica & Bozzoni 2014, Guttman & Rinn 2012). LncRNA SOX21-AS1 aggravates the oxidative stress injury through elevating FZD3/5 by regulating the

Wnt/β-catenin signaling pathway in mice with Alzheimer's disease (Zhang et al. 2019). LncRNA FLRL2 regulates lipogenesis, endoplasmic reticulum (ER) stress, and inflammation via the Arntl-sirt1 pathway in nonalcoholic fatty liver disease (Chen et al. 2019). Previous studies have confirmed that lncRNA KCNQ1OT1 was implicated in the pathogenesis of various diseases (Li et al. 2017, Yang et al. 2018, Yu et al. 2019, Zhang et al. 2020). Importantly, KCNQ1OT1 has been reported to regulate inflammatory response. In detail, KCNQ1OT1 knockdown significantly ameliorates the inflammation and cell apoptosis of cardiomyocyte induced by oxygen-glucose deprivation/re-oxygenation (Yi et al. 2020). KCNQ1OT1 regulates cardiac microvascular endothelial cell apoptosis and inflammatory response (Wang et al. 2019). KCNQ1OT1 ameliorates the liver injury induced by acetaminophen through the regulation of cell apoptosis and inflammatory response (Pei et al. 2020). Accordingly, we predicted that KCNQ1OT1 might participate in the regulation of inflammation and cell apoptosis in SCI.

In this work, we intended to uncover the regulatory mechanism of KCNQ1OT1 in SCI, and the key finding of our study was that KCNQ1OT1 promoted the inflammatory response and apoptosis of microglia after SCI by regulating the miR-589-5p/NPTN axis, which suggests that KCNQ1OT1 might serve as a potential therapeutic target for SCI.

MATERIALS AND METHODS

Animals

Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). Animal study was performed based on the Guide for the Care and Use of Laboratory Animals of the Chinese National Institutes of Health with the approval of the Animal Care Committee of The First People's Hospital of Lianyungang (Jiangsu, China). Eight-week-old C57BL/6 male mice (n=70, weight 20–25 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). A controlled environment with a temperature of 22–24°C, relative humidity of 40%, and a 12 h light-dark cycle was provided for all mice. All the mice had free access to food and water.

Mouse SCI model establishment

To induce spinal cord injury, mice were operated as previously described (Hara et al. 2017). In brief, mice were received the intraperitoneal injection of pentobarbital sodium (50 mg/kg; Sigma-Aldrich, USA) before laminectomy for anesthesia. Then, an incision along the medial dorsal line was made to expose the posterior vertebral arches from T8 to T12. Laminectomy was performed with a length of 3 mm under dissection stereomicroscope. For spinal cord crush operation, the spinal cord surface was struck with a 10 g weight dropping from a height of 5 cm. In the sham group, all the mice were only subject to laminectomy. Mice were sacrificed by cervical dislocation after intraperitoneal injection of sodium pentobarbital (50 mg/kg) on day 1, 3 and 28.

Basso, Beattie, and Bresnahan (BBB) score

Neurological function of the mice was evaluated by use of the Basso, Beattie, and Bresnahan (BBB) scoring according to the motor ability post SCI (Xu et al. 2017). BBB scores show a 21-point open field locomotor scale, where 0 represents complete immobility and 21 normal motor functions. Double-blinded observation of the locomotion of mice was performed at 1, 3, 7, 14, 21, and 28 day after SCI. The hind limb movements, stepping, position, paw placement, trunk stability, toe clearance, coordination and

tail position of the mice were observed and recorded.

Lentivirus (LV) construction

On day 1 and 3, mice in sham and SCI group (n=5/group/day) were sacrificed by cervical dislocation after sodium pentobarbital injection. Then, mice in SCI group were injected with LV-3 (GenePharma, Shanghai, China). In detail, the sequence of short hairpin RNA (shRNA) targeting KCNQ1OT1 (sh-KCNQ1OT1) with negative control sequence (sh-NC), miR-589-5p, and NPTN were separately ligated into the LV-3 (pGLVH1/GFP + Puro) vector. The viruses were packaged in HEK-293FT cells (ATCC, Manassas, VA, USA) according to the standard protocols and harvested after 72 h. Three days after SCI, 10 μL of packaged lentivirus was injected in situ with a microsyringe. Same dose of empty virus served as control. All mice were sacrificed on day 28.

Microglia culture and treatment

Isolation of microglia from injured spinal cord was performed 3 days after SCI as described (Gingras et al. 2007). Microglia were cultured in a humid incubator containing Dulbecco's Modified Eagle's medium (DMEM; GIBCO) with 10% calf serum (GIBCO), 5% fetal bovine serum (FBS; GIBCO), and 1% penicillin-streptomycin (HyClone) with 5% CO_2 at 37°C.

Terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL)

On day 1, 3 and 28, spinal cord tissues were isolated, paraffin-embedded and sliced into slides (5 μm-thick) for TUNEL staining. In short, tissue slides were dewaxed, rehydrated, and incubated with Proteinase K (20 μg/mL) at room temperature. After 15 min, the slides were washed with PBS thrice and incubated in TUNEL reaction mixture in dark for 60 min at 37°C. The nuclei were then stained for 5 min at room temperature. The immunofluorescent labeling was observed using a fluorescence microscope. The fluorescence intensity was evaluated by ImageJ software.

RNA immunoprecipitation (RIP) assay

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, MA, USA) was used for RIP assay following the manufacturer's protocol. Briefly, microglia (3×10^6) were lysed in polysome lysis buffer, and then the supernatant was immunoprecipitated with protein A/G magnetic beads containing AGO2 antibody overnight at 4°C. Magnetic bead-bound complexes with AGO2 were immobilized, and the RNAs were extracted from complexes for RT-qPCR analysis.

Luciferase reporter assay

KCNQ1OT1-Wt or KCNQ1OT1-Mut vector was co-transfected, respectively, with NC mimics or miR-589-5p mimics into BV2 cells. NPTN-Wt or NPTN-Mut was co-transfected, respectively, with NC mimics or miR-589-5p mimics into BV2 cells. Lipofectamine 2000 was used for transfection. After two days, the luciferase activity was evaluated adopting luciferase reporter assay system.

Enzyme linked immunosorbent assay (ELISA)

On day 1, 3 and 28, the content of tumor necrosis factor-α (TNF-α), interleukin- (IL-) 1β, and IL-6 in the mouse serum and microglia supernatants was detected by ELISA kits (R&D Systems, USA) following the manufacturer's instruction. A microplate reader (BioTek, USA) was used to measure absorbance at 450 nm.

RT-qPCR

Total RNAs were collected from microglia or spinal cord tissues using TRIzol reagent (Invitrogen) and then reverse transcribed into

cDNAs with a Reverse Transcription Kit (Toyobo, Osaka, Japan). SYBR-Green Real-Time PCR Kit (Takara, Dalian, China) was used to perform PCR. The mRNA levels of genes were calculated with the $2^{-\Delta\Delta Ct}$ method, with GAPDH and U6 as internal controls.

Western blot analysis

Total protein was extracted from microglia or spinal cord tissues prepared in radioimmunoprecipitation assay lysis buffer (Beyotime, Haimen, China), and loaded in 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred on PVDF membranes (Millipore, Billerica, MA, USA) by electrophoresis. The membranes were blocked with 5% defatted milk and then incubated with primary antibodies including anti-Bax (ab182734, 1/1000, Abcam), anti-Bcl-2 (ab196495, 1/1000, Abcam), anti-Cleaved Caspase-3 (ab49822, 1/500, Abcam) at 4°C overnight. Then, the PVDF membranes were incubated with secondary antibodies for 1 h (ab6721, 1:5000, Abcam). The enhanced chemiluminescence detection kits (Advansta, Wuhan, China) were used to visualize the target protein signals. The protein bands were quantified by Image software (BioRad, Hercules, CA, US).

Data analysis

The results are presented as the mean ± SD. Statistical significance between 2 or more groups were assessed using Student's *t*-test or analysis of variance followed by Tukey's *post hoc* test. Pearson's correlation analysis was adopted to analyze the expression correlations among target genes. $p < 0.05$ was considered statistically significant.

RESULTS

SCI induced cell apoptosis and inflammation

A mice model of SCI injury was constructed to more deeply investigate the pathogenesis of SCI (Hara et al. 2017). After the SCI model was successfully established, the behavioral analyses of mice were measured. First, Basso, Beattie, and Bresnahan (BBB) scores for hindlimb locomotion was evaluated, showing the BBB scores were strikingly decreased in SCI group compared with those in the sham group (Figure 1a). As the occurrence of apoptosis is a vital marker of SCI (Li et al. 1996), we then examined the apoptosis in SCI group and sham group using TUNEL assay. The results disclosed that more TUNEL positive cells were observed in the SCI group 3 days post-injury than those in the sham group (Figure 1b). In addition, from the data of western blot, we found that Bax and Cleaved-caspase-3 protein levels were increased while Bcl-2 protein level was decreased in the SCI group 3 days post-injury compared to the sham group (Figure 1c). In addition, excessive inflammatory response is another marker of secondary injury after SCI (Bethea et al. 1998), thus we next evaluated the proinflammatory cytokines (IL-6, TNF-α and IL-1β) in the serum of SCI mice. Results from ELISA delineated that IL-6, TNF-α and IL-1β concentrations were markedly elevated in the SCI mice compared with those in the sham group (Figure 1d-1f). Overall, SCI induced cell apoptosis and inflammation.

Silencing of KCNQ1OT1 limited the apoptosis and inflammatory response *in vivo*

Furthermore, the role and function of KCNQ1OT1 in SCI mice were explored. First, the expression of KCNQ1OT1 was determined by RT-qPCR. From the data, we observed that KCNQ1OT1 expression was significantly enhanced in the SCI group. Then, LV-shRNA targeting KCNQ1OT1 was injected

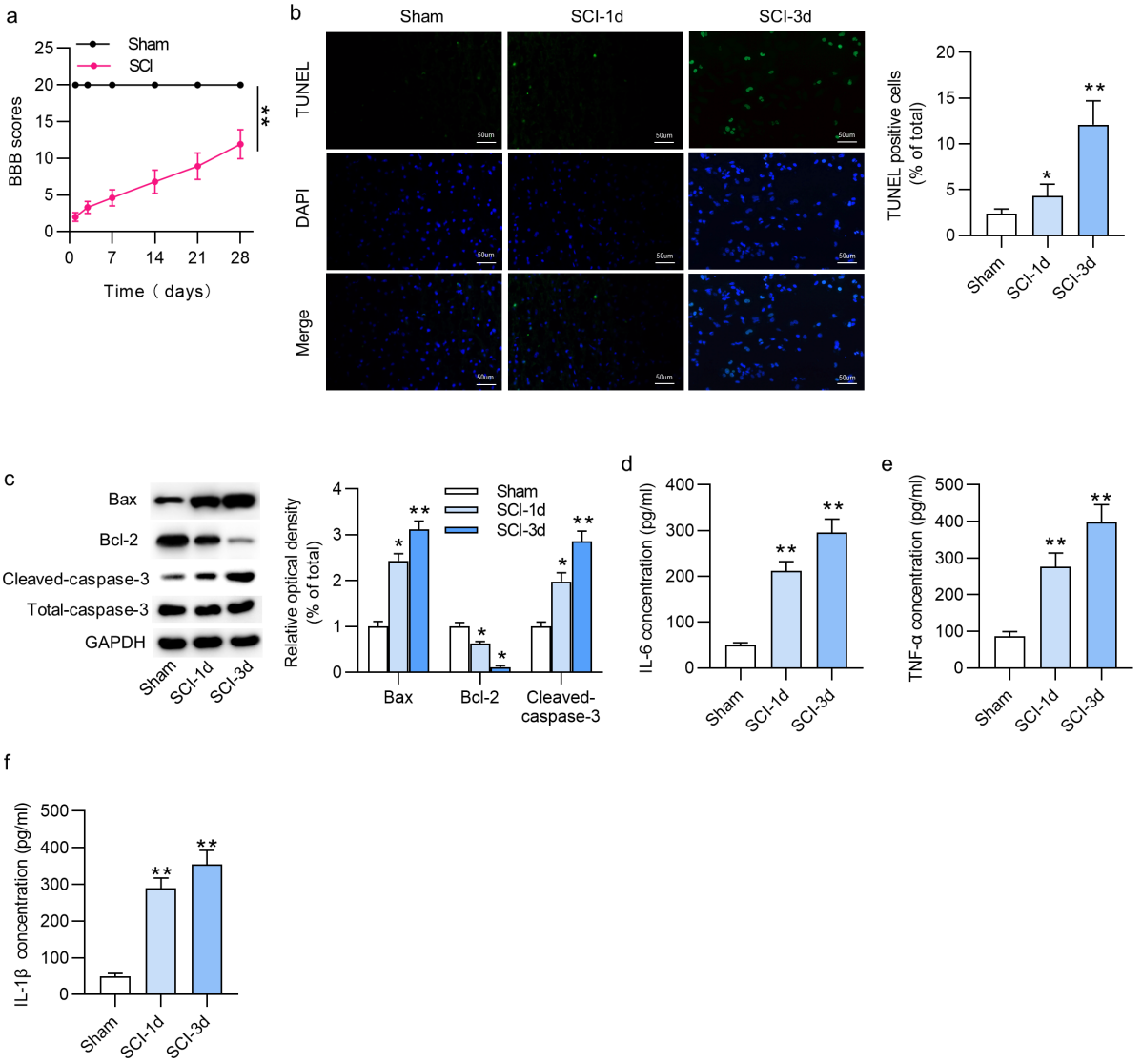

Figure 1. SCI induced the increase of the cell apoptosis and inflammation. (a) The behavioral analyses of the mice were measured via Basso, Beattie, and Bresnahan (BBB) scores. (b) Cell apoptosis in the SCI group and sham group was examined by use of TUNEL assay. (c) Western blot analysis was employed to evaluate the protein levels of apoptosis associated genes (Bax, Bcl-2 and Cleaved-caspase-3). (d-f) ELISA was performed to detect the concentrations of proinflammatory cytokines (IL-6, TNF-α and IL-1β) in the mouse serum. N=5 in each group. $^{*}p < 0.05$. $^{**}p < 0.01$.

into the mice to downregulate the expression of KCNQ1OT1 in the mice (Figure 2a). As shown in Figure 2b the mouse locomotor activity was improved in KCNQ1OT1 downregulated group relative to the SCI + sh-NC group. In addition, TUNEL staining revealed that the number of TUNEL positive cells were decreased due to the downregulation of KCNQ1OT1 (Figure 2c-2d). Consistently, suppression of KCNQ1OT1 reduced the protein levels of Bax and Cleaved-caspase-3 but increased the protein level of Bcl-2 (Figure 2e-2f), hinting KCNQ1OT1 deficiency inhibited cell apoptosis in the SCI mice. Additionally, the cytokine concentration was also decreased in SCI + sh-KCNQ1OT1 group (Figure 2g-2i). In summary, silencing of KCNQ1OT1 attenuated the apoptosis and inflammatory response *in vivo*.

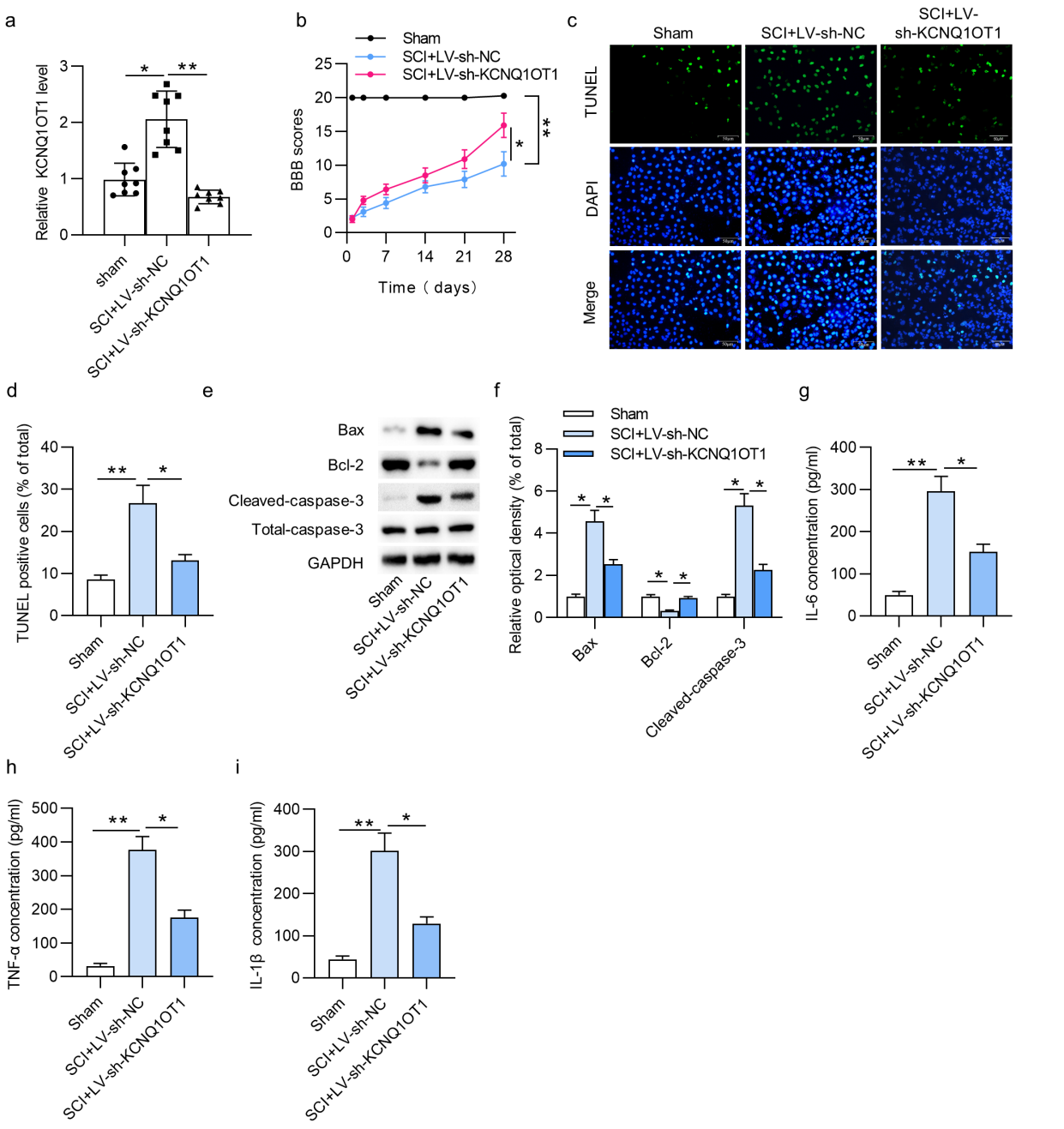

Figure 2. Silencing of KCNQ1OT1 alleviated apoptosis of microglia and inflammatory response. (a) RT-qPCR analysis was carried out to detect KCNQ1OT1 level after injection of LV-shRNA targeting KCNQ1OT1 into mice. (b) BBB scores showed the behavioral analyses of the mice. (c-d) TUNEL staining was used to test the number of TUNEL positive cells in different groups. (e-f) The protein levels of Bax, Bcl-2 and Cleaved-caspase-3 in different groups were determined by western blot analysis. (g-i) ELISA was used to measure the concentrations of IL-6, TNF-α and IL-1β in different groups. N=8 in each group. $^{*}p < 0.05$. $^{**}p < 0.01$.

KCNQ1OT1 bound with miR-589-5p

Considering lncRNAs have been proposed to act as competitive endogenous RNAs (ceRNAs) for miRNAs (Tay et al. 2014), we hypothesized that KCNQ1OT1 might regulate SCI progression via a ceRNA pattern. The starbase website (http://starbase.sysu.edu.cn/) was used to find the potential miRNAs sponged by KCNQ1OT1, and three candidate miRNAs (miR-589-5p, miR-146a-5p and miR-98-5p) were found. RT-qPCR exhibited that only miR-589-5p was downregulated in the SCI-3d group (Figure 3a). Then, miR-589-5p was chosen for the following exploration. An RIP assay showed that KCNQ1OT1 and miR-589-5p were enriched in the Ago2 group rather than the IgG group (Figure 3b), suggesting KCNQ1OT1 and miR-589-5p were in the same RNA-induced silencing complex (RISC). Then we effectively overexpressed miR-589-5p in microglia from the SCI mice via transfection of miR-589-5p mimics (Figure 3c). The binding site between KCNQ1OT1 and miR-589-5p was exhibited in Figure 3d. Luciferase reporter assays showed that miR-589-5p mimics significantly weakened the luciferase activity of the KCNQ1OT1-Wt vector instead of the KCNQ1OT1-Mut vector (Figure 3e). Subsequently, we measured miR-589-5p level in spinal cord tissues and found that miR-589-5p expression was downregulated in SCI group (Figure 3f). Thereafter, miR-589-5p expression was overexpressed by LV-miR-589-5p injected into the SCI mice (Figure 3f). In addition, Pearson's expression correlation analysis unveiled KCNQ1OT1 level was negatively correlated with miR-589-5p level in the spinal cord tissues of the SCI mice (Figure 3g). MiR-589-5p overexpression markedly increased the locomotor activity of the mice in miR-589-5p overexpression group compared with that in the SCI group (Figure 3h). In addition, miR-589-5p upregulation also attenuated cell apoptosis and proinflammatory cytokine concentrations in SCI mice (Figure 3i-3m). In conclusion, KCNQ1OT1 bound with miR-589-5p, and miR-589-5p overexpression inhibited the apoptosis and inflammatory response.

NPTN was a target of miR-589-5p

Next, the potential target of miR-589-5p was investigated. Bioinformatics analysis showed that 7 mRNAs (NPTN, TRAF6, SORT1, ABL2, CAVIN1, PAPPA and BBX) had potential binding site for miR-589-5p (Figure 4a). RT-qPCR analysis demonstrated that NPTN expression was significantly decreased upon transfection of miR-589-5p mimics (Figure 4b). The enrichment of miR-589-5p and NPTN was observed in the Ago2 group but was not significant in the IgG group (Figure 4c). The potential binding site between miR-589-5p and NPTN was exhibited in Figure 4d. Luciferase reporter assays showed the luciferase activity of the NPTN-Wt vector was overtly decreased by miR-589-5p overexpression while the luciferase activity of the NPTN-Mut vector showed no significant change between the two groups (Figure 4d). Additionally, NPTN presented high level in SCI group (Figure 4e). Moreover, NPTN expression was negatively associated with miR-589-5p expression but positively associated with KCNQ1OT1 expression (Figure 4f-4g). In summary, NPTN was a target of miR-589-5p.

KCNQ1OT1 promoted the apoptosis and inflammatory response of microglia by regulating NPTN in SCI

Subsequently, we focused on whether KCNQ1OT1 regulated SCI by the modulation of NPTN. First, LV-NPTN was injected into the mice to overexpress NPTN, showing an increase of NPTN protein level in the SCI mice (Figure 5a). Results of TUNEL assay showed that overexpression of NPTN reversed the inhibitory effect of KCNQ1OT1 downregulation on TUNEL positive cells in the

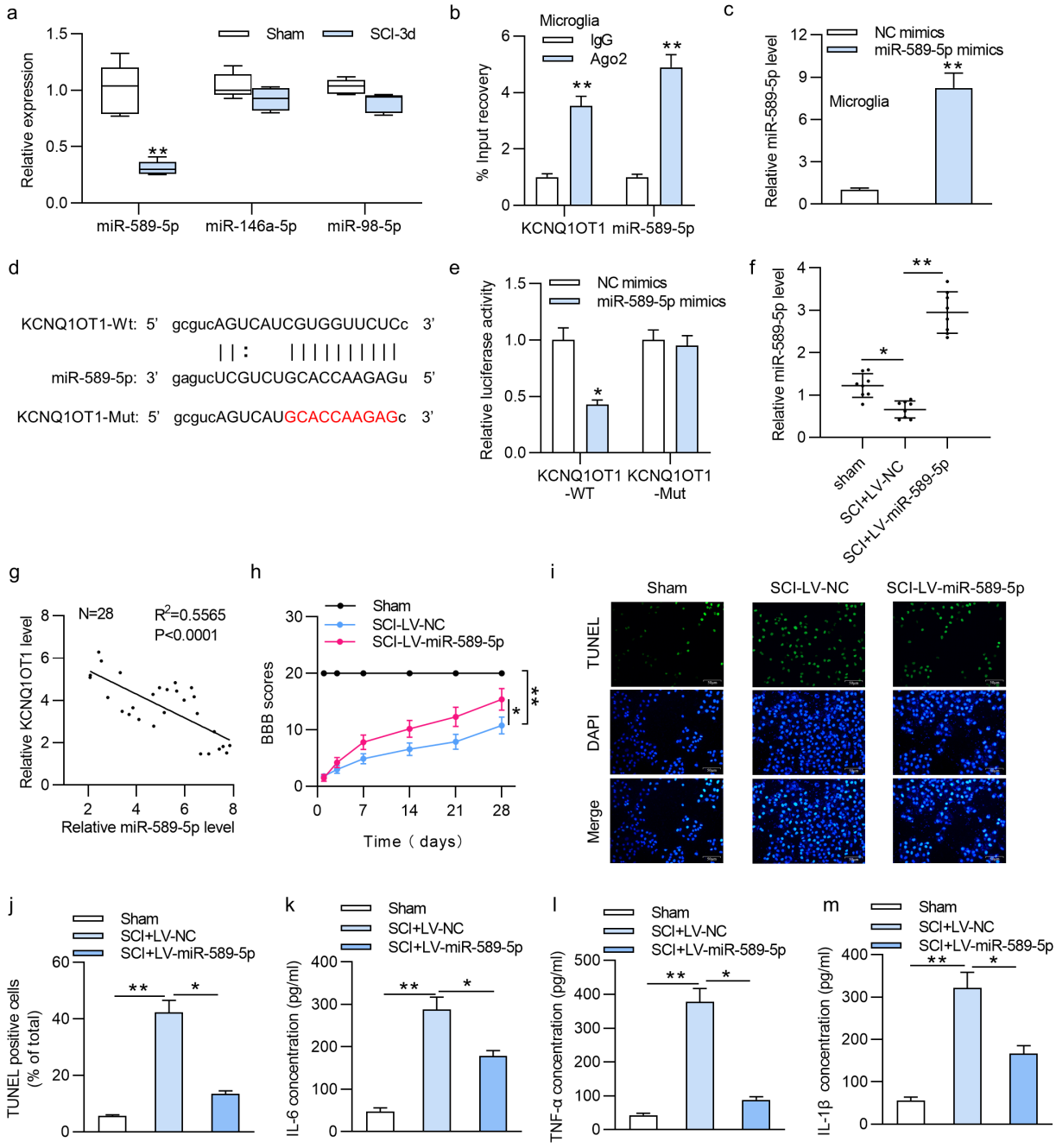

Figure 3. KCNQ1OT1 bound with miR-589-5p. (a) The expression of miR-589-5p, miR-146a-5p and miR-98-5p in SCI mice was detected by RT-qPCR. (b) RIP revealed KCNQ1OT1 and miR-589-5p were in the same RNA-induced silencing complex (RISC). (c) RT-qPCR displayed the expression of miR-589-5p in microglia from the SCI mice after transfection of miR-589-5p mimics. (d) The putative binding site between KCNQ1OT1 and miR-589-5p was exhibited. (e) Luciferase reporter assay showed KCNQ1OT1 bound with miR-589-5p. (f) RT-qPCR delineated miR-589-5p expression after LV-miR-589-5p injection into SCI mice. (g) Pearson's correlation analysis unveiled the correlation between expression of miR-589-5p and KCNQ1OT1 expression in SCI mice. (h) BBB scores was used to analyze the neurological function of the mice. (i-j) The number of TUNEL positive cells in different groups was measured by TUNEL staining. (k-m) ELISA was used to measure the concentrations of IL-6, TNF-α and IL-1β in different groups. N=8 in each group for h-m. $^{*}p < 0.05$. $^{**}p < 0.01$.

SCI mice (Figure 5b-5c). The decreased Bax and Cleaved-caspase-3 protein levels as well as the increased protein level of Bcl-2 in the SCI mice resulting from KCNQ1OT1 silencing were abolished by NPTN overexpression (Figure 5d). Eventually, the inhibited effects of LV-sh-KCNQ1OT1 on IL-6, TNF-α and IL-1β concentration were rescued by NPTN overexpression (Figure 5e). In conclusion, KCNQ1OT1 promoted the apoptosis and inflammatory response by regulating NPTN in SCI.

DISCUSSION

Spinal cord injury (SCI) is a grievous disease that can lead to paralysis and therefore diminish the patient quality of life. After SCI, neurons are seriously damaged, thus the occurrence of cell apoptosis is a vital marker of SCI (Li et al. 1996).

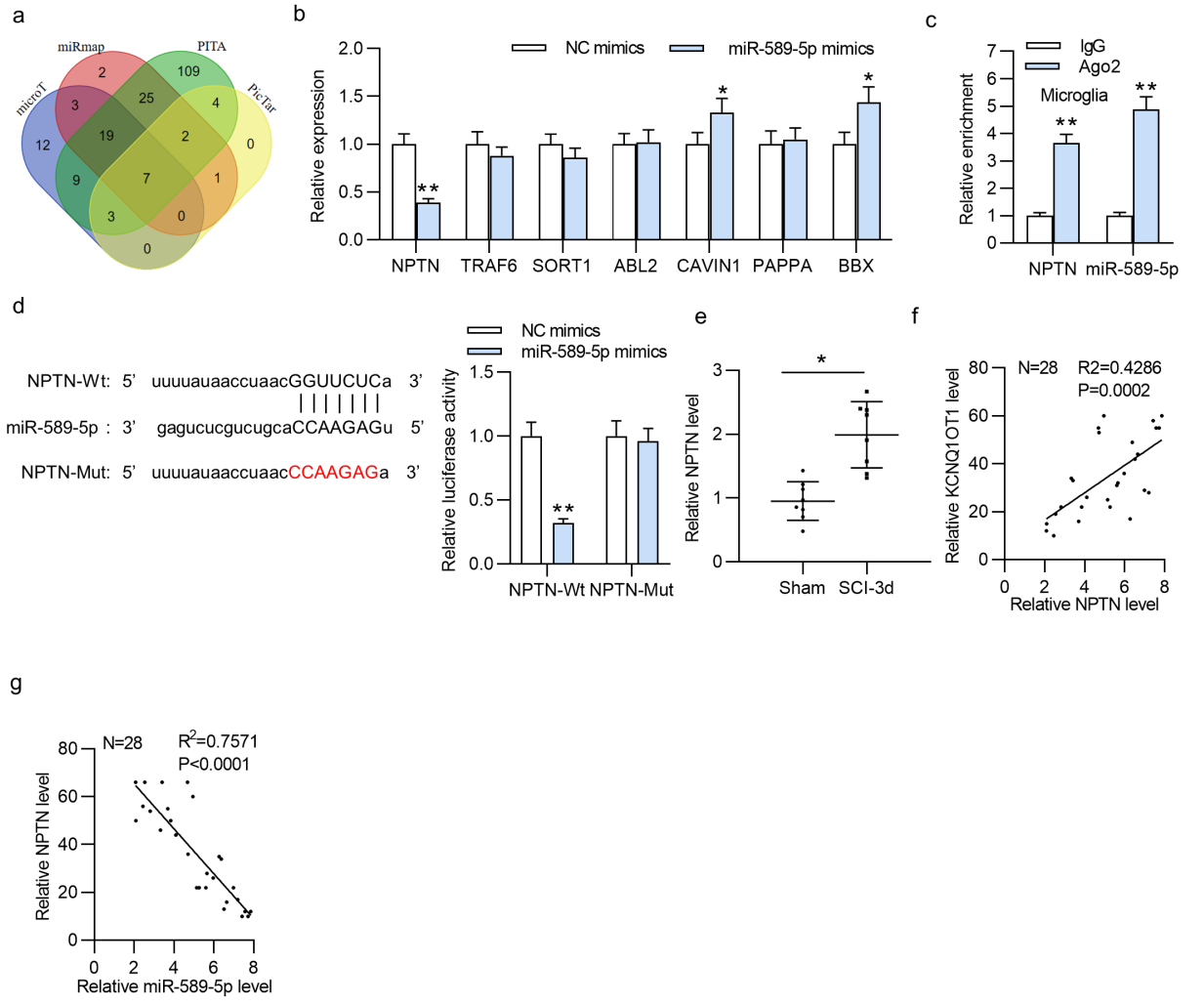

Figure 4. **NPTN was a target of miR-589-5p. (a) Bioinformatics analysis was performed to find the potential targets of miR-589-5p. (b) The expression of the candidate mRNAs upon miR-589-5p mimics was detected by RT-qPCR. (c) RIP indicated the enrichment of miR-589-5p and NPTN in Ago2 and IgG group. (d) The potential binding site between NPTN and miR-589-5p was exhibited. Luciferase reporter assay showed miR-589-5p bound with NPTN. (e) NPTN level in spinal cord tissues. (f-g) Pearson's correlation analysis showed the correlation between NPTN and miR-589-5p as well as NPTN and KCNQ1OT1. $^{*}p < 0.05$. $^{**}p < 0.01$.**

Therefore, the prevention of cell apoptosis after SCI may help improve the spinal tissue repair and motor function (Crowe et al. 1997). Nonetheless, it is still not fully elucidated about the exact mechanisms of such apoptosis in SCI. In addition, excessive inflammatory response is proposed to be another marker for secondary injury after SCI (Bethea et al. 1998). Excessive inflammatory response has been reported to increase apoptosis and further aggravate secondary injury (Bank et al. 2015). Therefore, our study focused on mechanisms associated with the apoptosis of microglia and inflammatory response after SCI. In current study, we studied

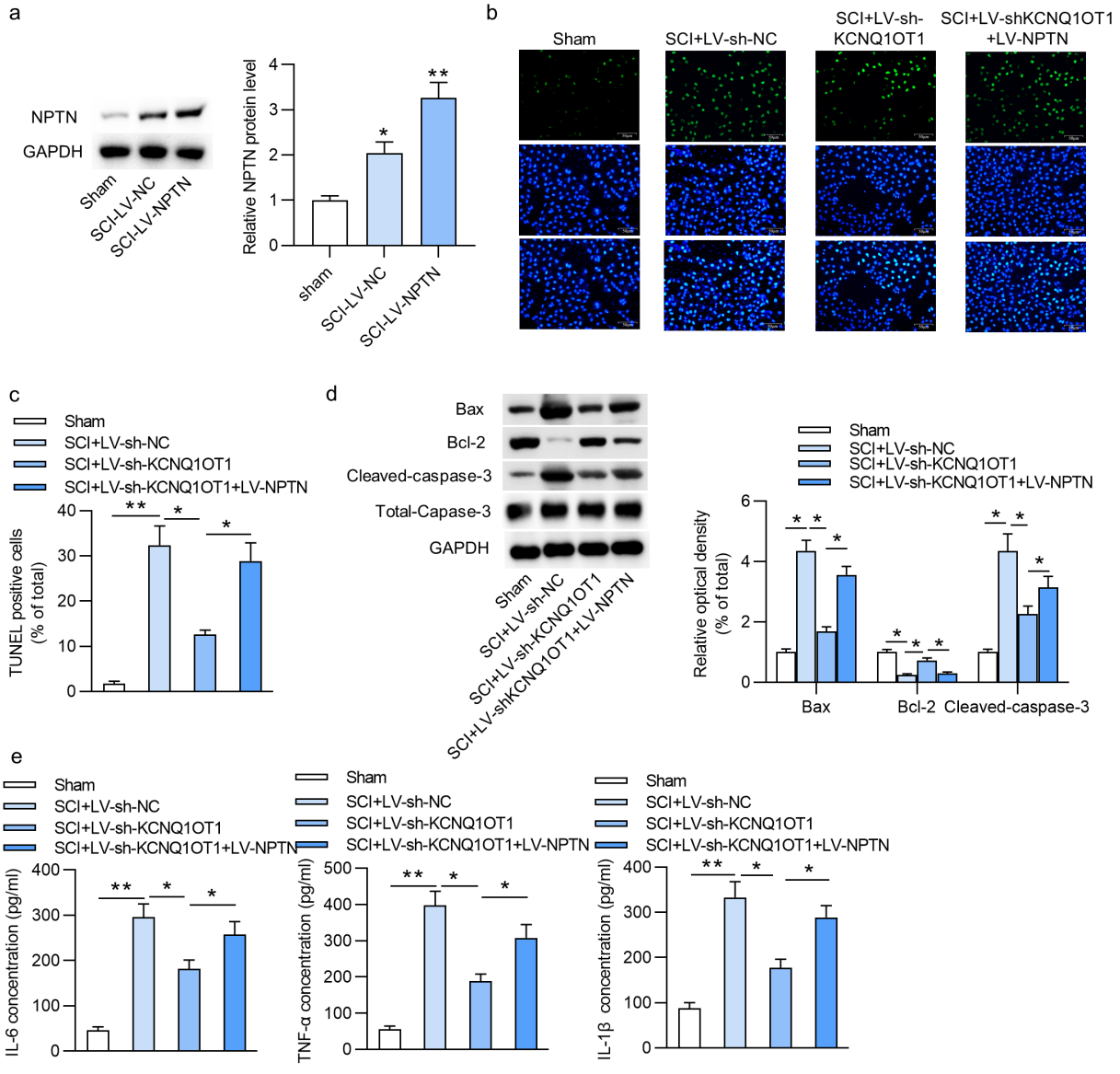

Figure 5. KCNQ1OT1 promoted the apoptosis of microglia and inflammatory response by regulating NPTN in SCI. (a) RT-qPCR and western blot analyses disclosed the mRNA and protein levels of NPTN in SCI mice after injection of LV-NPTN. (b-c) The number of TUNEL positive cells in different groups was assessed by TUNEL staining. (d) The protein levels of Bax, Bcl-2 and Cleaved-caspase-3 in SCI mice were detected by western blot analysis. (e) ELISA measured the concentrations of IL-6, TNF-α and IL-1β in different groups. N=8 in each group. $^{*}p < 0.05$. $^{**}p < 0.01$.

the role and function of KCNQ1OT1 after SCI. The results delineated that the KCNQ1OT1 expression was significantly enhanced in the SCI group. Moreover, silencing of KCNQ1OT1 alleviated the apoptosis of microglia and inflammatory response. In summary, KCNQ1OT1 played a significant part after SCI.

Recently, ample studies have revealed that lncRNAs can modulate the expression of messenger RNAs (mRNAs) via acting as competing endogenous RNAs (ceRNAs) to competitively bind to microRNAs (miRNAs) (Tay et al. 2014). Therefore, we suspected that lncRNA KCNQ1OT1 might regulate SCI in the same pattern. MiRNAs are short noncoding RNAs that regulate gene expression via combining with the 3′-untranslated region (3′UTR) of mRNAs (Matoulkova et al. 2012). In this study, bioinformatics analysis predicted the binding site for KCNQ1OT1 and miR-589-5p. MiR-589-5p was previously reported to be sponged by lncRNA FABP5P3 and target ZMYND19 in hepatocellular carcinoma cell proliferation, migration and invasion (Zhu et al. 2018). However, whether KCNQ1OT1 could sponge miR-589-5p to regulate SCI was still elusive. Present study verified that KCNQ1OT1 bound with miR-589-5p. Moreover, miR-589-5p expression was notably decreased in SCI mice. Pearson's correlation analysis unveiled the miR-589-5p level was negatively correlated with KCNQ1OT1 level in SCI mice. Additionally, miR-589-5p upregulation also attenuated cell apoptosis and proinflammatory cytokine levels in SCI mice. In conclusion, KCNQ1OT1 bound with miR-589-5p to regulate SCI.

Neuroplastin (NPTN) has been reported to participate in cell apoptosis and inflammatory response in melanoma (Sumardika et al. 2018). Nonetheless, the specific role of NPTN in SCI was rarely studied. In this study, we identified that miR-589-5p could target NPTN. Additionally, the expression of NPTN was conspicuously elevated in SCI mice. Moreover, rescue assays showed that KCNQ1OT1 promoted cell apoptosis and inflammatory response by regulating NPTN in SCI.

In summary, this study corroborated that KCNQ1OT1 promoted apoptosis of microglia and inflammatory response by regulating the miR-589-5p/NPTN axis after SCI, which suggests KCNQ1OT1 may be valuable in the treatment of SCI.

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