A novel Tweak/ Stat1/ Snhg3 positive feedback circuit contributes to the glial cell activation in temporal lobe epilepsy mice

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Abstract

Gliosis is an important feature of temporal lobe epilepsy (TLE), but the regulatory mechanism of glial cell activation remains unclear. Small nucleolar RNA host gene 3 (Snhg3) has been reported to be involved in cell proliferation and migration in various cancers. However, its role in the development of TLE has been hardly explored. Here, we established a mouse TLE model by injecting intraperitoneally with pilocarpine, and found that Tweak expression was significantly induced in TLE mice. Inhibiting Tweak expression by the injection of siRNA notably rescued the glial cell activation, neuroinflammatory cytokine secretion and cognitive behavior disorder in TLE mice. Molecular mechanism studies showed that cell proliferation, migration, inflammatory factor secretion, Stat1 pathway activation and Snhg3 expression were promoted after we incubated Tweak recombinant protein (rTweak) with mouse astrocytes (MAs). The Tweak neutralizing antibody (anti-Tweak) showed the opposite effect to that of rTweak. In subsequent researches, we found that Stat1 directly bound to Snhg3 promoter and they elevated the expression of each other. Moreover, both of them boosted cell proliferation, migration, inflammatory factor secretion and Tweak expression. Thus, we found a feedback regulation loop consisting of Tweak/Fn14, Stat1/ p-Stat1 and Snhg3 in the MAs, which increased cell activation. In vivo experiment demonstrated that the reduction of Snhg3 inhibited glial cell activation induced by TLE, and Tweak/Stat1/Snhg3 feedback circuit also existed in TLE mice. In short, our research testified a feedback regulation loop consisting of Tweak/Stat1/Snhg3, which was involved in the activation of hippocampal glial cells in TLE mice.

Introduction

Temporal lobe epilepsy (TLE) is the most common intractable epilepsy in adults [1]. It originated from or involved the internal structure of the temporal lobe such as the hippocampus [1]. Typical pathological features of temporal lobe epilepsy include massive proliferation of glial cells, significant loss of neurons, and abnormal mossy fiber sprouting [2]. TLE is usually accompanied by decreased cognitive ability and memory deficits in patients [3]. The activation and proliferation of glial cells can cause inflammatory reactions, imbalance of intracellular ions and neurotransmitters, and loss of blood-brain barrier, and lead to excessive discharge of neurons, thereby promoting the development of epilepsy [4–6]. However, the mechanism of glial cell activation is complex and has not been fully explored [6].

Tumor necrosis factor (TNF)-related weak inducer of apoptosis (TWEAK) is a member of the tumor necrosis factor superfamily[7]. It is involved in a variety of biological effects, including cell proliferation, differentiation, migration, apoptosis and necrosis, and induction of inflammatory cytokine expression [7, 8]. The main receptor for TWEAK is Fn14, a fibroblast growth factor inducible 14 kDa protein, and the main source of TWEAK protein are monocyte/macrophage family cells, including microglia [9]. Soluble TWEAK can modulate the expression of proteins that are involved in inflammation and opening of the blood brain barrier [9]. In murine and human astrocytes, the TWEAK/Fn14 pathway stimulates reactivity when cell proliferation is activated and inflammatory factors are produced [10]. TWEAK/Fn14 pathway is involved in human neurodegenerative disease. Anti-TWEAK antibodies can improve a murine model of parkinson's disease [11].

LncRNA is a class of non-coding RNAs greater than 200 nucleotides in length. LncRNAs play important roles in chromatin remodeling, transcriptional regulation and post-transcriptional processing [12, 13]. Recent studies have found that LncRNAs are involved in the development and progression of epilepsy. It has been reported that LncRNA UCA1 inhibits epilepsy and seizure-induced brain injury by regulating miR-495/Nrf2-ARE signal pathway [14]. LncRNA H19 contributes to apoptosis of hippocampal neurons by inhibiting let-7b in a rat TLE model [15]. Small nucleolar RNA host gene 3 (Snhg3) is considered to be involved in cell proliferation in various cancers [16]. It has been reported that LncRNA Snhg3 is up-regulated in the brain of the AD11 transgenic mouse model expressing NGF resistance, and its up-regulation may be an indicator of AD11 neurodegenerative progression disorder [17].

STAT1 is a member of the STATs protein family and is composed of seven potential cytoplasmic transcription factors that play key roles in different physiological functions including apoptosis, cell survival, immune response, and proliferation [18]. It has been demonstrated that TWEAK induces proinflammatory genes mRNA expression through its receptor Fn14 and STAT1 activation in cultured vascular smooth muscle cells [19]. Researches have shown that LncRNA H19 contributes to hippocampal glial cell activation via JAK/STAT signaling in a rat model of temporal lobe epilepsy [20]. In this study, we found a feedback regulation loop consisting of Tweak/Stat1/Snhg3, which was involved in the activation of hippocampal glial cells in mice with temporal lobe epilepsy.

Materials And Methods

Epilepsy model

Sixty adult male mice weighing 25-30 g were used in this study. All animals were placed in a room where temperature and humidity were controlled (18-25 °C and 50-60%, respectively), with a light/dark cycle of 12 hours, water and food were freely available. This study was approved by the Ethics Committee of the Shaanxi Provincial People's Hospital.

Mice were injected intraperitoneally with pilocarpine (300 mg/Kg) and scopolamine (1 mg/Kg) was injected 20 minutes before the injection of pilocarpine to reduce the peripheral effects of pilocarpine [21]. The severity of seizures was graded using the Racine scale: class 1, facial convulsions; class 2, head nodding; class 3, forelimb clonus; class 4, bilateral forelimb clonus and rearing; and class 5, rearing and falling. One hour after the onset of grade 4-5 seizures, diazepam (10 mg/Kg) was used to terminate seizure. If the animals didn't develop 4-5 grade seizures after 30 minutes of pilocarpine injection, they will be excluded. The control mice were injected with the same amount of normal saline.

Cell transfection

Mouse astrocytes (MAs) were purchased from Shanghai Institute of Cell Biology (Shanghai, China). All of the cells were cultured with 10% FBS (Hyclone, Logan, UT, USA) at 37 °C in a humidified 5% CO₂ atmosphere.

The siRNAs of Snhg3, Stat1, Fn14 and negative control siRNAs were synthesized by Invitrogen Carlsbad (CA, USA), recombinant protein and neutralizing antibody of Tweak were purchased from GenePharma Co. (Shanghai, China). For the construction of overexpression vectors, full-length cDNA of Snhg3 and full-length CDS of Stat1 were inserted into the pcDNA3.1 empty vector. When the cell density reached about 70% confluence, the vectors or siRNAs were transfected into astrocytes by using the Lipofectamine® 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Cell proliferation

Each group of cells was seeded in a 96-well plate and cultured at 37 °C, 5% CO₂ for 12, 24, 48, and 72 hours, respectively. Then, CCK-8 solution (10 µL) was added at each time point, after which the cells were further cultured for 4 hours, and cell proliferation was expressed by the OD value at 450 nm.

Transwell assay

The transfected cells were resuspended in 200 µL serum-free DMEM and seeded in a 24-well transwell upper chamber with a density of 5×10^4 cells per well. A total of 1 mL DMEM containing 10% FBS was added to the lower chamber. After incubation for 48 hours in an incubator at 37 °C, the cells in the upper chamber were removed, and the cells invading in the lower chamber were fixed with formaldehyde.

Tweak and Snhg3 interference *in vivo*

After induction of TLE for 30 days, the Tweak and Snhg3 siRNAs (1 mg/Kg) or NC siRNAs (1 mg/Kg) were injected into the right lateral ventricle (0.3 mm posterior, 1.0 mm lateral, and 2.5 mm ventral to bregma) of mice once daily for 3 d [21].

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the fresh tissues or cultures by using TRIZOL (Thermo Fisher Scientific, Waltham, MA, USA), and reversely transcribed into cDNAs according to the manufacturer's instructions. The RNA expression was detected by using a SYBR ExScript qPCR kit (Takara, Dalian, China) with the following conditions: 95 °C for 3 min, followed by denaturation at 94 °C for 15 s, annealing at 55 °C for 25 s and extension at 72 °C for 15 s for 35 cycles. Each test was performed three times independently. Relative quantification was calculated with the $2^{-\Delta\Delta CT}$ formula. 18S RNA was used as internal control.

Western blotting

RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) was used to isolate proteins from fresh tissues or cultures. The protein samples were separated by polyacrylamide gels and then electro-transferred onto polyvinylidene fluoride membranes (Millipore, Boston, MA, USA). Primary antibodies used in the study including anti-Snhg3 (1:400 dilution; Abcam, Cambridge, UK), anti-Tweak (1:400 dilution; Abcam, Cambridge, UK), anti-Stat1 (1:400 dilution; Abcam, Cambridge, UK), anti-p-Stat1 (1:400 dilution; Abcam, Cambridge, UK), anti-Fn14 (1:400 dilution; Abcam, Cambridge, UK), anti-β-Actin (1:400 dilution; Abcam,

Cambridge, UK), anti-OX42 (1:400 dilution; Abcam, Cambridge, UK). After incubation with antibodies at 4 °C overnight, membranes were incubated with HRP-conjugated secondary antibodies at 37 °C for 1 h. Detect the protein abundances by a ChemiDoc XRS Imaging System (Bio-Rad, Hercules, CA, USA).

Enzyme linked immunosorbent assay (ELISA)

The secretions of IL-1 β , IL-6, TNF- α in cerebrospinal fluid and cell culture supernatant were detected by ELISA kits (Sigma, St. Louis, MO, USA) following the manufacturer's instructions.

Morris water maze test

The water maze test was based on previous studies [22]. If the mice did not find the platform within 120 seconds, their escape latency was recorded as 120 seconds and placed them on the platform allowed to rest for 30 seconds. On the 30th day, the spatial probe test without the platform was evaluated and the number of times the mouse was placed through the platform in 120 seconds was recorded.

Prediction of binding manners between Snhg3 and Stat1

We analyzed the promoter of Snhg3 by CHIPBase (<http://rna.sysu.edu.cn/chipbase/>), predicted the binding sites of Stat1 and Snhg3.

Luciferase reporter gene assay

Inserting different segments cloned from Snhg3 into pGL3-basic vector (Promega, Madison, WI, USA) and the Stat1 sequence was connected upstream. These vectors were transfected into the mouse astrocytes after correct sequencing, and samples were collected 48 hours later. Luciferase activity was detected using the double luciferase reporter assay system.

Chromatin immunoprecipitation (ChIP) assay

We first cross-linked the cells with formaldehyde and then quenched reaction by glycine fixative, next lysed the cells, and disrupted them with sonication. ChIP-IT Expression Chromatin Immunoprecipitation Kits (Active motif, Carlsbad, CA, USA) was used in CHIP assay. Incubate with the above complex using the Stat1 antibody and normal serum IgG overnight at 4 °C. Twenty percent chromatin that was not incubated with the antibody was used as the input. Finally, the complex with beads were eluted according to the instructions and semi-quantitative analysis was performed.

Statistical analysis

Data analysis was performed using SPSS version 22.0, and every experiment was repeated at least three times. All data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical differences between the two groups were compared using Student's t test or two-way ANOVA, and the difference was considered significant, $P < 0.05$.

Results

Tweak played a role in the activation of glial cells in TLE mice.

To investigate the role of Tweak in glial cell activation, a mouse model of TLE was constructed, and then Tweak siRNA and its control (NC siRNA) were injected into the hippocampus of successfully modeled mice. We first verified whether the epilepsy model was established with success. The results showed that the protein expression of GFAP and OX42 in mouse hippocampus and the expression levels of inflammatory factors including IL-1 β , IL-6 and TNF- α in mouse cerebrospinal fluid were significantly elevated compared with the control (Fig. 1B). Moreover, the escape latency of the mice was significantly prolonged after the induction of TLE (Fig. 1C). These data indicated successful modeling and activation of astrocyte and microglia. Next, the Tweak expression in the TLE group was observably induced, and the injection of Tweak siRNA observably inhibited the up-regulation expression of Tweak (Fig. 1A, B). We also found that compared with the TLE group, the expression of GFAP and OX42 and the protein levels of IL-1 β , IL-6 and TNF- α were decreased after the inhibition of Tweak (Fig. 1B). What's more, the escape latency of mice was also obviously shortened (Fig. 1C). As a result, Tweak was involved in the glial cell activation in TLE mice.

Overexpression of Tweak in MAs promoted cell proliferation and migration, Snhg3 expression, and Stat1 pathway activation.

Mouse astrocytes (MAs) were applied for exploring the regulatory mechanisms by which Tweak involved in glial cell activation. After transfection of different concentrations of Tweak recombinant protein, the expression of inflammatory factors including IL-1 β , IL-6 and TNF- α in the supernatant was prominently increased compared with the control (Fig. 2B-D). Cell proliferation and migration was significantly elevated after incubated by Tweak recombinant protein (Fig. 2E, F). Next, we tested the effect of Tweak on Stat1 signaling pathway, and the results displayed that both Stat1 and phosphorylated Stat1 expression were elevated, and the expression of c-Myc gene downstream of Stat1 was prominently inhibited compared with the control (Fig. 1G). The expression of Fn14 which was the Tweak receptor and LncRNA Snhg3 were also up-regulated in the Tweak group (Fig. 1H, I). Furthermore, all of the above expression changes were dose dependent. In brief, Tweak was overexpressed in astrocytes, which accelerated cell activation and Snhg3 expression, activated the Stat1 pathway.

The activation of MAs and Stat1 pathway, the expression of Snhg3 were inhibited after interfering Tweak.

To more accurately explore the regulatory mechanisms that Tweak involved, we transfected Tweak neutralizing antibodies into MAs and tested for cell proliferation, migration and expression changes of the above-mentioned genes. The results indicated that compared with the control, the cell proliferation and migration was distinctly decreased (Fig. 3B, C), and the expression of the three inflammatory factors, Stat1, p-Stat1 and c-Myc were inhibited (Fig. 3D-G) in Tweak inhibition group. Moreover, the expression of Fn14 and Snhg3 was also notably down-regulated when Tweak interfered (Fig. 3H, I). In short, MAs and Stat1 pathway activation, and Snhg3 expression were declined by the inhibition of Tweak.

Stat1 directly bound to Snhg3 promoter.

Since there was no direct evidence that Stat1 interacted with Snhg3, we analyzed the promoter region of Snhg3 with CHIPBase and found a potential binding site of Stat1. The predicted binding region was 379 bp upstream of the transcription initiation site. To verify the binding efficiency of Stat1 to the Snhg3 promoter, we constructed several luciferase reporter vectors containing the Stat1 sequence and different truncated sequences of Snhg3 (Fig. 4A). There was little difference in luciferase activity between the reporter without the binding region and the empty vector group (Fig. 4B). However, the luciferase activity of the reporter vector containing only the binding region was not significantly different from that of the full-length sequence, and both luciferase activities were notably increased compared to the control (Fig. 4B). The CHIP assay was used to further investigate the binding relationship between Stat1 and Snhg3. As a result, it was found that the IgG, NC group did not precipitate the Snhg3 promoter compared to the input, and the expression of Snhg3 promoter was significantly enriched in the Stat1 antibody-precipitated protein-nucleotide complex (Fig. 4C). It indicated that Stat1 could directly bind to the promoter of Snhg3.

To explore the regulatory mechanisms that Stat1 involved in glial cells, Stat1 overexpression vector and siRNA were transfected into MAs. As shown, after overexpression of Stat1, the expression levels of Tweak, Fn14, and Snhg3 were observably increased compared with the control (Fig. 5B, C). Moreover, the IL-6 and TNF- α were also markedly up-regulated (Fig. 5D, E). After inhibiting Stat1 with siRNA, Snhg3 was significantly down-regulated, indicating that Stat1 can directly regulate the expression of Snhg3 (Fig. 5C). However, the expression of Tweak, Fn14, IL-6 and TNF- α showed a downward trend after inhibiting Stat1, but it was not significant (Fig. 5B, D-E). This might be because p-Stat1 did not change after Stat1 was interfered, and p-Stat1 continued to function.

Tweak/Fn14, Stat1/p-Stat1, Snhg3 formed a regulation circuit.

Previous studies have revealed that Tweak could promote the expression of Stat1, p-Stat1 and Snhg3, while Stat1 not only promoted the expression of Snhg3, but also facilitated the expression of Tweak. Next, we explored the regulation mechanisms that Snhg3 involved. The expression of Tweak, Fn14, Stat1, p-Stat1 and inflammatory factors was significantly increased in the Snhg3 overexpression group and down-regulated in the Snhg3 inhibition group compared with the respective control groups (Fig. 6B, C and F-H). Moreover, after overexpressing Snhg3, the proliferative and migratory capacity of MAs were stronger than control; after interfering Snhg3, it displayed opposite trends. (Fig. 6D, E). As a result, Tweak/Fn14, Stat1/p-Stat1, Snhg3 formed a positive feedback loop in MAs.

To further verify the existence of the Tweak/Stat1/Snhg3 feedback recruit, 100 ng/mL recombinant Tweak protein was used to incubate with MAs alone, or together with siRNAs against Fn14, Stat1 or Snhg3. The results revealed that, compared with the rTweak group, the expression levels of Tweak, Fn14, Stat1, p-Stat1 and Snhg3, cell proliferation, migration and inflammatory factor secretion were all declined in the group which rTweak was co-treated with siRNAs against Fn14, Stat1 or Snhg3 (Fig. 7A-G). However, after rTweak was incubated with Fn14 siRNA, the expression levels of Tweak, Stat1, p-Stat1 and Snhg3,

cell proliferation, migration and inflammatory factor secretion all showed a downward trend compared with the rTweak group, but there was no statistical significance (Fig. 7A-G). These results demonstrated the predecessor's research that Tweak regulated downstream gene expression through Fn14 which was the Tweak receptor. In brief, Tweak/Fn14, Stat1/ p-Stat1, Snhg3 formed a positive feedback loop in MAs.

Tweak/ Stat1/ Snhg3 regulation circuit also existed in TLE mouse model.

It had been demonstrated that, Tweak/ Stat1/ Snhg3 regulation loop was present in MAs. We next investigated whether such regulatory mechanism was also present in in vivo model. Snhg3 siRNA and its negative control were injected into the hippocampus of successfully modeled mice. The expression of Snhg3 was significantly induced in TLE mice, while injection of siRNA inhibited the Snhg3 expression (Fig. 8A). The expression of Tweak, Fn14, Stat1 and p-Stat1 in TLE group was significantly increased, whereas Snhg3 interference could resist these impacts (Fig. 8B). Compared with the TLE group, the increased expression of GFAP, OX42 and inflammatory factors, and the prolonged mouse escape latency were rescued after interference with Snhg3 (Fig. 8C-D). In short, the inhibition of Snhg3 could reduce the activation of mice glial cells, and there was also a Tweak/Stat1/ Snhg3 regulatory loop in TLE mice.

Discussion

Astrocytes (ASTs) was involved in the regulation of extracellular ions and neurotransmitters, and buffering defects in extracellular ion changes could lead to epilepsy [23]. Recent researches had confirmed that vascular pathological changes were referred to the development of epilepsy, and AST played an important role in this process [24]. Epilepsy was closely related to excitatory and inhibitory neurotransmitter imbalances in the central nervous system, and the stimulatory amino acid transporter (EAAT) on the AST cell membrane played a vital role [6]. Studies had shown that pro-inflammatory factors could alter the connections between glial cells and nerve cells, leading to epilepsy and neurologic damage associated with epilepsy [25]. Moreover, ASTs were the main source of inflammatory signals for epileptogenesis. In this study, we selected mouse astrocytes as a model to demonstrate the feedback loop consisted of Tweak/Stat1/Snhg3, and found that this loop promoted the secretion of inflammatory factors and increased cell viability.

Increased neuronal excitability and synaptic remodeling are important pathophysiological changes in epilepsy. LncRNAs may regulate the occurrence and development of epilepsy through various mechanisms such as neurogenesis, neurotransmitter regulation, ion channel and synaptic plasticity [26]. Researches have shown that absence of certain LncRNAs could result in abnormal development of the nervous system [27]. Dlx5 regulated the expression of adjacent homologous genes, thereby modulating neuronal differentiation [28]. The number of GABAergic interneurons in the hippocampus and dentate gyrus of Evt2-deficient mice was reduced, leading to excessive neuronal excitation, and causing epilepsy [29]. Lack of LncRNA BC1 increased neuronal excitability and promoted epileptogenesis [30]. Small nucleolar RNA host genes have been shown to be involved in the development and progression of some neurological diseases. There has been shown that LncRNA SNHG1 promotes neuroinflammation in

Parkinson's disease via regulating miR-7/NLRP3 pathway [31]. LncRNA SNHG14 promotes inflammatory response induced by cerebral ischemia/reperfusion injury through regulating miR-136-5p/ROCK1 [32]. It has been reported that LncRNA Snhg3 up-regulation may be an indicator of AD11 transgenic mouse model neurodegenerative progression disorder [17]. Our study displayed that Snhg3 was obviously induced in the TLE mouse model, and the elevated expression of GFAP, OX42, and inflammatory factors and the prolongation of escape latency in mice caused by TLE were rescued after interference with Snhg3, showing that LncRNA Snhg3 was involved in the progression of TLE.

STAT1 plays important roles in some nervous system diseases. Researches have shown that STAT1 deficiency markedly exacerbated the pathophysiological actions of IFN- α in the central nervous system [33]. The increased NF- κ B and STAT1 α in cell nuclei might be involved in inflammatory activation in Alzheimer's disease (AD) brains [34]. Excessive activation of signal transducer and STAT1 and successive induction of iNOS expression were involved in the etiology of brain injury associated to ischemia/reperfusion (I/R) and neurodegenerative diseases [34]. Studies also show that a rapid and sustained seizure-induced activation of STAT3 and STAT1 was observed exclusively in reactive astrocytes in the hippocampus [35]. Our findings were consistent with previous reports, Stat1/p-Stat1 were up-regulated in glial-activated TLE mouse model. Stat1 directly bound to Snhg3 promoter and they could elevate the expression of each other. Moreover, Stat1 was also regulated by Tweak.

The TWEAK/Fn14 pathway has been shown to be involved in chronic human inflammatory pathologies such as neurodegeneration, autoimmunity or malignant diseases [10]. TWEAK/Fn14 can modulate neuroinflammation by activating the canonical and non-canonical NF- κ B pathways, as well as by stimulating mitogen-activated protein kinase signaling [10]. TWEAK/Fn14 mediates atrial-derived HL-1 myocytes hypertrophy via JAK2/STAT3 signalling pathway [36]. It has been demonstrated that TWEAK induces proinflammatory genes mRNA expression through its receptor Fn14 and STAT1 activation in cultured vascular smooth muscle cells [19]. Tweak/Fn14 has been shown to be highly expressed in many cancer tumors, and could regulate several pivotal events related to tumor progression and metastasis, including immune surveillance and angiogenesis [37, 38]. We demonstrated that Tweak was sharply induced in a TLE mouse model and could reduce the activation of glial cells after interfering with it, and Tweak could up-regulate the expression of Snhg3 and Stat1/p-Stat1 both in MAs and TLE mouse model.

In this study, we found that astrocytes and microglia in TLE mice were activated, and then Tweak expression was instantly induced. Tweak interference significantly rescued the glial cell activation, inflammatory factor secretion and cognitive behavior disorder of TLE mice. The results of the mechanism investigation revealed the presence of Tweak/Stat1/Snhg3 regulatory loop in vitro. Further investigation showed that inhibition of Snhg3 was able to inhibit the activation of glial cells induced by TLE, and Tweak, Stat1 and Snhg3 interacted in a TLE mouse model as well. In conclusion, our research testified a feedback regulation circuit consisting of Tweak/Stat1/Snhg3, which was involved in the activation of hippocampal glial cells in TLE mice.

Declarations

Abbreviations

TLE: Temporal lobe epilepsy; ASTs: astrocytes; Snhg3: LncRNA Snhg3; rTweak: Tweak recombinant protein; anti-Tweak: Tweak neutralizing antibody.

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Authors' contributions

LC designed and analyzed the experiment, and was a major contributor in writing the manuscript. RS, XZ, CH, XHZ, LC, RY and RL performed the experiment. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Shaanxi Provincial People's Hospital. All experiments were in accordance with the guide for the care and use of laboratory animals established by United States National Institutes of Health (Bethesda, MD, USA).

Consent for publication

Not applicable.

Conflict of Interests

There is no conflict of interest to be declared by the authors.

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Figures

Figure 1

Tweak played a role in glial cells activation in TLE mice. A TLE mouse model was established by injecting intraperitoneally with pilocarpine(300 mg/kg), and siRNA specifically antagonizing Tweak and negative control siRNA were injected into the right lateral ventricle (0.3mm posterior, 1.0mm lateral, and 2.5mm ventral to bregma) once daily for 3 d. The successful modeling group was utilized as TLE. The mRNA expression of (A) Tweak was detected by qPCR. The protein expression of (B) Tweak, GFAP, OX42, IL-1β, IL-6, TNF-α was detected by Western blotting. (C) Water maze test showed alteration of mouse escape latency. *P < 0.05, **P< 0.01.

Figure 2

Overexpression of Tweak in MAs promoted cell proliferation and migration, Snhg3 expression and Stat1 pathway activation. 10ng/mL and 100ng/mL recombinant Tweak protein was incubated with MAs for 24 h. A. The overexpression efficiencies of Tweak were detected with Western blotting. The protein levels of (B) IL-1β, (C) IL-6, (D) TNF-α in the supernatant were detected with ELISA. E. Cell migration was detected by Transwell assay. F. Cell proliferation was detected with CCK-8. And the protein alteration of (G) Stat1, p-Stat1, c-Myc and (H) Fn14 was showed by Western blotting. I. The mRNA expression of Snhg3 was detected by qPCR. *P < 0.05, #P < 0.05 versus 10ng/mL Tweak group.

Figure 3

The activation of MAs and Stat1 pathway, the expression of Snhg3 were inhibited after interfering Tweak. Tweak neutralizing antibody was incubated with MAs for 24 h. A. The interference efficiencies of Tweak were detected with Western blotting. B. Cell migration was detected by Transwell assay. C. Cell proliferation were detected with CCK-8. And the protein levels of (D) IL-1β, (E) IL-6, (F) TNF-α in the supernatant were detected with ELISA. The protein alteration of (G) Stat1, p-Stat1, c-Myc and (H) Fn14 was showed by Western blotting. I. The mRNA expression of Snhg3 was detected by qPCR. *P < 0.05, **P < 0.01.

Figure 4

Stat1 directly bound to Snhg3 promoter. Through the ChIPBase online bioinformatics server, the potential binding pattern between Stat1 and Snhg3 was analyzed. And the binding capacity and binding relationship between them were respectively validated by luciferase reporter assay and ChIP assay. A. Graphical representations of Stat1 binding regions' detailed information. Stat1 binding regions were indicated in blue and the four exons of Snhg3 were presented in orange, green, red, wathet and with the yellow arrow representing the direction of transcription. The red bases highlighted Stat1 binding motif. B. The luciferase reporter assay showed the relative luciferase activities of the reporters. These reporters consisted of different sequences of the Stat1 binding regions, which were located upstream of the luciferase reporters and downstream of Stat1. The Stat1 binding regions of Snhg3 were indicated in blue. C. Anti-Stat1 was used to perform ChIP experiments, and the input chromatin template (20%) served as positive control and IgG-precipitated template as a specificity control. The enrichment of Snhg3 promoter sequence was detected with RT-PCR. **P< 0.01.

Figure 5

Stat1 promoted the expression of Snhg3, Tweak and Fn14. Stat1 overexpression vectors and Stat1 siRNAs were transfected into MAs, following transfection for 48 h, A. the Stat1 overexpression and inhibition efficiencies were detected with Western blotting. The protein levels of (B) Tweak, Fn14 and the mRNA expression of (C) Snhg3 were showed by Western blotting and qPCR. And the expression of (D) IL-6, (E) TNF-α were detected by ELISA. *P < 0.05, **P< 0.01.

Figure 6

Snhg3 promoted the expression of Tweak, Fn14, Stat1 and p-Stat1. Snhg3 overexpression vectors and Snhg3 siRNAs were transfected into MAs, after transfecting for 48 h, A. the overexpression and interference efficiencies were detected with q-PCR. The expression of (B) Tweak, Fn14, (C) Stat1, p-Stat1

were detected by Western blotting. (D) Cell proliferation was detected with CCK-8. E. Cell migration was detected by Transwell assay. And the protein levels of (F) IL-1 β , (G) IL-6, (H) TNF- α in the supernatant were detected with ELISA. *P < 0.05, **P < 0.01.

Figure 7

Tweak/Fn14, Stat1/p-Stat1, Snhg3 formed a regulation circuit in MAs. 100 ng/mL recombinant Tweak protein was used to incubate with MAs alone, or together with siRNAs against Fn14, Tweak or Snhg3 for 24h, and (A) Tweak, Fn14, Stat1, p-Stat1 protein and (B) Snhg3 mRNA were detected by Western blotting and q-PCR. C. Cell migration was detected by Transwell assay. Next, (D) Cell proliferation were detected with CCK-8, and the expression of (E) IL-1 β , (F) IL-6, (G) TNF- α in the supernatant were detected with ELISA. *P < 0.05, #P < 0.05 versus 100 ng/mL Tweak group.

Figure 8

Tweak/Fn14, Stat1/ p-Stat1, Snhg3 regulation circuit also existed in TLE mice. Snhg3 siRNA and its negative control were injected into the right lateral ventricle (0.3mm posterior, 1.0mm lateral, and 2.5mm ventral to bregma) once daily for 3 d. A. The mRNA expression of Snhg3 was detected by qPCR. The protein levels of (B) Tweak, Fn14, Stat1 and p-Stat1, (C) GFAP, OX42, IL-1β, IL-6 and TNF-α were detected by Western blotting. (D) Water maze test showed alteration of mouse escape latency. *P < 0.05, **P < 0.01.