Long non-coding RNA NEAT1 aggravates epilepsy and seizure-induced neuronal damage by regulating microRNA-139/ROCK1 axis

Xiaowan Li

Qilu Hospital of Shangdong University

Fang Hao

Liaocheng People's Hospital

Shuxin Tao

Liaocheng People's Hospital

Weihua Wang

Liaocheng People's Hospital

Xinxing Xiao

Liaocheng People's Hospital

Dong Guo

Liaocheng People's Hospital

Hongling Ma

Liaocheng People's Hospital

Xuewu Liu (✉ xuewu2333@163.com)

Liaocheng People's Hospital <https://orcid.org/0000-0002-8210-4510>

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Abstract

Background

Both long non-coding RNA (lncRNA) NEAT1 and microRNA (miR)-139 are crucial gene regulators in various disorders. This study aims to investigate their role in epilepsy and seizure-induced neuronal damage.

Methods

In this research, rat model of epilepsy was established by pilocarpine induction. The RNA and protein expression in hippocampal tissues and neurons were determined by RT-qPCR and Western blot analysis, respectively. Microarray analysis was used to predict the relationship between NEAT1 and miR-139 or between miR-139 and ROCK1, and dual luciferase reporter gene assay was performed to verify the interaction. The endogenous expression of related genes was modulated by recombinant plasmids and cell transfection. The cell apoptosis, levels of inflammatory factors and cell proliferation were detected by flow cytometry, ELISA and EdU assay.

Results

lncRNA NEAT1 and ROCK1 was upregulated, while the miR-139 was downregulated in hippocampal tissues and neurons of epileptic rats. Overexpression of NEAT1 decreased the activity of neurons, increased cell apoptosis, and increased the level of inflammatory factors. NEAT1 negatively targeted miR-139 to upregulate ROCK1. The RhoA/ROCK1 signaling pathway was activated by NEAT1 overexpression and miR-139 downregulation.

Conclusion

lncRNA NEAT1 suppressed pilocarpine-induced epilepsy by inhibiting apoptosis of hippocampal neurons through miR-139/RhoA/ROCK1 axis, and thereby inhibiting neuronal injury induced by seizure.

Background

As one of the most prevailing neurological syndromes, epilepsy influences over 50 million individuals globally [1]. The principal feature linked with epilepsy is intense and frequent aberrant emission of brain neurons, clinically manifested with dysfunction of transient central nervous system (CNS) [2]. Unrestricted inflammatory reactions might induct abnormal neuronal bond and hyper-excitable neuronal system, accelerating the initiation and development of epilepsy [3]. The mechanisms of epileptogenesis is deliberated to cover the dysfunction of genes contributing to the progression of neuronal signaling, cell

death and inflammation [4]. Although a lot of researches dedicated to experimenting the pathogenesis of epilepsy, the definite causative elements and specified mechanisms of epilepsy are still mysterious.

Long noncoding RNAs (lncRNAs) have been validated to control the growth and behavior of the CNS, and lncRNAs dysregulation may result in destruction of the brain [5, 6]. Contemporary studies have determined that specific lncRNAs are closely linked with epilepsy. For instance, evidence provided by Li et al emphasizes that lncRNA GAS5 silencing obstructed the onset and progression of epilepsy [2]. Findings of Wu et al indicated that lncRNA MALAT1 down-regulation could protect hippocampal neurons against apoptosis and autophagy through promoting the PI3K/Akt signaling pathway in rats with epilepsy [7]. lncRNA nuclear-enriched abundant transcript 1 (NEAT1) is a recently observed crucial factor of nuclear paraspeckles, which has been validated to perform as an oncogene in assorted human cancers, such as pancreatic cancer, colon cancer, glioma and hepatocellular carcinoma [8–11]. It was noticed that NEAT1 also plays a role in brains and linked to multiple cognitive and neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease [12, 13]. In an in vivo model of generalized seizures, the complete transcriptional program was initiated by NEAT1-dependent activities after neuronal activity in the hippocampus [14]. Above all, we may hypothesize that NEAT1 may play a part in epilepsy-induced neuronal apoptosis and inflammation.

In the present study, we observed NEAT1 expression was evidently increased in the hippocampal tissues and neurons of epilepsy rats. We, therefore studied the role of NEAT1 in epilepsy-induced hippocampal apoptosis and inflammation, and researched the possible downstream targets of NEAT1 such as microRNA (miR) and mRNA in hippocampal neurons, with the intention of revealing the mechanism of NEAT1 in the occurrence and development of epilepsy and providing a potentially practicable new direction for the clinical treatment.

Methods

Ethics statement

All the animal related experiments were permitted by the Ethics Committee of Liaocheng People's Hospital and accomplished according to the Chinese Animal Welfare Act. Efforts were made to lessen the number of animals used and their aching.

Pilocarpine-induced epilepsy in rats

A total of 30 Sprague-Dawley rats (21 d, 80 ± 20 g) were purchased from the Charles River (Beijing, China). All rats were housed in a suitable environment on a 12-h light/dark cycle and allowed to drink and eat freely. After 5 weeks of feeding, the rats were prepared for further experiment. The rats were assigned into control group ($n = 3$) and epilepsy group ($n = 27$). To establish the epilepsy model, lithium chloride (125 mg/kg; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was intraperitoneally injected 16 h prior to atropine administration (1 mg/kg, intraperitoneal injection; Wuhan Boster Biological Technology Co., Ltd., Hubei, China). After that, pilocarpine (15 ng/kg; Dalian Meilun Biotechnology Co., Ltd., Liaoning, China)

was intraperitoneally injected into rats to induce status epilepticus (SE). After 30 min, seizures were terminated by intraperitoneal injection of 1% pentobarbital sodium (40 mg/kg). Subsequently, the rat behavior was observed after pilocarpine injection. On the basis of the Racine's scale, the rats identified with stage IV or above were as successful models and selected for subsequent experiments.

Cell culture and treatment

Both human embryonic kidney 293T (HEK293T) cells and rat astrocytes CTX-TNA2 and DI TNC1 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) [15], which were seeded in Dulbecco's modified Eagles Medium containing 10% fetal bovine serum (FBS), 4 mM glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin and 4.5 g/L glucose under an atmosphere of 5% CO₂. CTX-TNA2 and DI TNC1 cells with stable growth were inoculated into 24 well plates and treated with interleukin (IL)-1β (10 ng/h) for 24 h for subsequent experiments. pCDNA3.1(+) plasmid was purchased from Kelei Bio. (Shanghai, China). NEAT1 mimic, miR-139 mimic and si-ROCK1 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The plasmid transfection was performed with the Lipofectamine 3000 transfection Kit (Invitrogen Inc., Carlsbad, CA, USA), and the results were verified by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Animal grouping and hippocampal tissues

Three rats without pilocarpine and atropine treatment were as normal group. Twenty seven epileptic rats were randomly divided into nine groups, three in each group: epilepsy group (epileptic rats without transfection), NEAT1 mimic group (epileptic rats transfected with NEAT1 mimic), NEAT1 control group (epileptic rats transfected with NEAT1 control), miR-139 mimic group (epileptic rats transfected with miR-139 mimic), miR-139 control group (epileptic rats transfected with miR-139 control), NMM group (epileptic rats transfected with NEAT1 mimic and miR-139 mimic), NM group (epileptic rats transfected with NEAT1 mimic), si-ROCK1 group (epileptic rats transfected with si-ROCK1), and ROCK1-NC group (epileptic rats transfected with ROCK1-NC). Epileptic rats were treated with intracerebroventricular injection and anesthetized with 1% pentobarbital sodium (40 mg/kg) intraperitoneally. The mouse head was fixed on the stereotaxic instrument, cut a 2-cm incision along the midline of the head to expose the "cross" bone structure of the anterior fontanelle. According to the rat stereoscopic map of Paxinos Watson's, the skull was drilled 1 mm behind the anterior fontanelle and 1.5 mm beside it. The microinjector was inserted vertically along the drill hole into the dura mater for 4.0 mm to inject RNA segment slowly. Then, the hole was sealed with dental cement, the incision was sewed with surgical suture, and the rat was fed in the cage regularly.

Hematoxylin-eosin (HE) staining

The rats were injected with 1% pentobarbital sodium (40 mg/kg) intraperitoneally for anesthesia. The rats were fixed in the supine position to open the chest and expose the heart. The syringe was inserted into the ascending aorta through the left ventricular. The right atrial appendage of the rat was cut open to infuse 4% paraformaldehyde into the heart until the heart turned white. Following the removal of all blood, the rats stopped autonomous breathing and heartbeat. After the confirmation of rat death, the cranium was

opened to extract the brain immediately [7]. The brains were fixed into Bouin's fixative solution for 24 h, dehydrated by gradient alcohol, put into dimethylbenzene, and embedded in paraffin. The hippocampal tissues were cut into the 5- μ m thick sections. The sections were then put into dimethylbenzene to dewax for 5 min, soaked in gradient concentration alcohol. After rinsing, the sections were put into hematoxylin dye solution (Leagene Bio., Anhui, China) for 12 min, immersed in 1% hydrochloric acid alcohol for 10 s, then transferred to eosin solution (Leagene Bio., Anhui, China) for 4 min. After that, the sections were dehydrated and cleared again, sealed with gum and observed under a microscope.

Microarray analysis

LncRNA microarray analysis was performed by Affymetrix (Santa Clara, CA, USA). Briefly, lncRNA was separately extracted using the TRIzol Reagent (Invitrogen Inc., Carlsbad, CA, USA) and purified with Rat LncRNA Array V3.0 (Arraystar). LncRNA was then labeled with Cy3 (000213, Thermo Fisher Scientific Inc., Waltham, MA, USA) and hybridized at 48 °C, 0.644 g to the gene chip. After washing and staining, the slides were scanned with the GeneChip™ Scanner 3000 7G Software for 16 h (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data analysis was conducted using Expression Console Software (version 1.3.1), including data normalization, aggregation, and quality control assessment. Robust multichip analysis was used to correct and normalize the original data. The differential expression of lncRNA was identified according to *t*-test analysis, $P < 0.05$ and $|\text{fold change}| > 1.5$ was defined as differential lncRNA, which was clustered by levels and plotted into heat map.

RNA isolation and quantitation

Total RNA from hippocampal tissues was isolated using TRIzol Reagent (Invitrogen Inc., Carlsbad, CA, USA). Reverse transcription was performed with PrimeScript™ reagent Kit (Takara Holdings Inc., Kyoto, Japan) for equal amounts of RNA to cDNA. The quantitative PCR was performed using the TaqMan™ Fast Advanced Master Mix on an QuantStudio 3 and 5 Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA) at the conditions of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 55°C for 20 s, followed by 72 °C for 30 s. Each sample was determined in triplicate. The relative expression was calculated with $2^{-\Delta\Delta C_t}$ method. The primers are presented in Supplementary Table 1.

5-ethynyl-2'-deoxyuridine (EdU) assay

Cell-Light EdU Apollo643 In Vitro Kit (100T, Guangzhou RiboBio Co., Ltd., Guangzhou, Guangdong, China) was used to conduct EdU assay. Cells of logarithmic growth stage were inoculated into a 96-well plate (1×10^4 cells/well) with 100 μ L, 50 μ M EdU culture medium for 2 h. After washed by phosphate buffer saline (PBS) for 5 min, the cells were incubated with 50 μ L PBS containing 4% paraformaldehyde for 30 min. After that, the cells were incubated with the corresponding staining solution in a shaker for 5 min, and added with 100 μ L penetrant (PBS containing 0.5% TritonX-100) for another 10-min incubation. Immediately after staining, the cells were observed under the fluorescence inverted microscope (Cx31, Olympus Optical Co., Ltd, Tokyo, Japan). Five visual fields were selected to take photos, and all the experiments are carried out three times independently.

Flow cytometry

A total of 1×10^6 cells were centrifuged for precipitate and suspended with 0.3 mL PBS containing 10% calf serum. Then, the fluid was transferred into a 1.5-mL eppendorf tube and fixed with 0.7 mL absolute ethanol at -20°C for more than 24 h. After that, the fluid was concentrated at 1610 g for 30 s with the supernatant removed. The cells were resuspended by 1 mL PBS with the supernatant removed again. The precipitated cells were suspended by 100 μL 1 mg/mL RNase A, and incubated with 400 μL 50 $\mu\text{g}/\text{mL}$ PI devoid of light for 10 min. The cell apoptosis was detected using the flow cytometer CytoFLEX (BECKMAN COULTER, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

After 48 h of transfection, the cells were inoculated in a 24-well plate (1×10^6 cells/well) for 24 h. The supernatant of cell culture medium was collected and centrifuged at 4°C for 1 min at 1800 g. Detection of tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β expression in supernatant was conducted using the rat ELISA Kits (ab100772, ab100785, ab100768, Abcam). The specific antibody globulin was diluted to 10 $\mu\text{g}/\text{mL}$, which was added to the plate (0.3 mL/well) and incubated at 4°C overnight. The cells were incubated with 0.2 mL diluted plasma at 37°C for 1 h, 0.2 mL diluted enzyme labeled antibody solution at 37°C for 1 h and 0.2 mL substrate at room temperature for 30 min. Finally, each well was added with 0.05 mL H_2SO_4 to terminate the reaction. The optical density (OD) value was determined with the enzyme marker (OPD = 492 nm).

Dual luciferase reporter gene assay

The 3'UTR sequences of NEAT1 binding to miR-139 and miR-139 binding to ROCK1 were predicted by online prediction software Starbase (<http://starbase.sysu.edu.cn/>). The 3'UTR of wild type (WT) and mutant (MUT) of NEAT1/miR-139 and miR-139/ROCK1 were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) and inserted into pMIR-REPORTTM (Thermo Fisher Scientific Inc., Waltham, MA, USA) Luciferase Report vector [16]. Using the Lipofectamine 3000 transfection Kit (Invitrogen Inc., Carlsbad, CA, USA), WT and MUT plasmids were co-transfected with NEAT1/miR-139 and miR-139/ROCK1 into cells, respectively. After 24 h, the cells were lysed. Luciferase activity intensity (intensity = RLU1/RLU2, RLU1 is the luciferase activity of firefly, RLU2 is the luciferase activity of renilla) was detected by dual luciferase reporter assay system (Promega Corporation, WI, USA) [16].

Western blot analysis

The hippocampal tissues were homogenized and then lysed (RIPA lysis buffer, pullair Gene Technology Co., Ltd.). After 30 min, the lysate was transferred to a 1.5-mL centrifuge tube with a pipette, and concentrated at 4°C at 25764 g for 5 min. The supernatant was treated with sodium dodecyl sulfate polyacrylamide gel electrophoresis and the gel obtained was transferred to the polyvinylidene fluoride membrane (EMD Millipore, Thermo Fisher Scientific Inc., Waltham, MA, USA). The membranes were blocked with 5% skim milk at room temperature and then incubated with primary antibodies against RhoA-GTP (1:1000, ab41435, Abcam), RhoA (1:5000, ab187027, Abcam), ROCK1 (1:3000, ab45171, Abcam), and β -actin (1:200, ab115777, Abcam) for 16 h. Subsequently, the membranes were incubated

with the secondary antibody (1:3000, ab205718, Abcam). The intensities of the protein bands were quantified with ImageJ software 1.8.0 (National Institute of Health, Md., USA).

Statistical analysis

All statistical analyses were performed using SPSS 21.0 (IBM Corp. Armonk, NY, USA). Data were in normal distribution according to Kolmogorov-Smirnov method and described as mean $\pm$ standard deviation. Differences among multiple groups were analyzed using one-way analysis of variance (ANOVA) or two-way ANOVA. Tukey's multiple comparisons test was used for the pairwise comparison after ANOVA analysis. p was obtained by two-tailed test and $p < 0.05$ was considered statistically significant.

Results

NEAT1 is highly expressed in the hippocampal tissues of epileptic rats

We observed the hippocampal tissues of epileptic rats and found that the pyramidal cells in the hippocampal tissues of epileptic rats were indistinct, the cell distance was too large and uneven, the number of pyramidal cells was significantly reduced, and the damage of hippocampal tissues was serious (Fig. 1A). We found that NEAT1 was abnormally overexpressed in the hippocampal tissues of epileptic rats by microarray analysis (Fig. 1B), and RT-qPCR verified this result (Fig. 1C). At the same time, the levels of TNF- α , IL-6 and IL-1 β were also found abnormally high in the hippocampal tissues of epileptic rats, which suggested that the occurrence of epilepsy activated the inflammation (Fig. 1D).

NEAT1 promotes neuronal damage

By detecting the CTX-TNA2 and DI TNC1 cells, we found that the IL-1 β content increased significantly, and epileptic cells were successfully constructed (Fig. 2A). We also found that cells treated with NEAT1 mimic showed higher NEAT1 expression compared with cells treated with NEAT1 control (Fig. 2B), and cells treated with NEAT1 mimic showed decreased neuronal viability (Fig. 2C) and increased apoptotic neurons (Fig. 2D). Furthermore, cells treated with NEAT1 mimic showed increased levels of IL-6 and TNF- α (Fig. 2E). Finally, we found that the epileptic rats showed aggravated symptoms and increased damaged hippocampal tissues after injection of NEAT1 mimic (Fig. 2F).

NEAT1 sponging miR-139 regulates ROCK1 expression

The target miRNA prediction of NEAT1 and dual luciferase reporter gene assay showed that NEAT1 bind to miR-139 (Fig. 3A). The expression of miR-139 in epileptic and normal rats was detected and the expression of miR-139 in epileptic rats was significantly down-regulated. Meanwhile, the CTX-TNA2 and DI TNC1 cells treated with NEAT1 mimic showed downregulated miR-139 compared with NEAT1 control treatment (Fig. 3B). The target mRNA prediction of miR-139 and dual luciferase reporter gene assay

showed that ROCK1 was one of the target gene of miR-139 (Fig. 3C). The expression of ROCK1 in epileptic cells was significantly up-regulated. Meanwhile, the CTX-TNA2 and DI TNC1 cells treated with miR-139 mimic showed upregulated ROCK1 compared with miR-139 control treatment (Fig. 3D).

Overexpression of miR-139 inhibits the biological effect of NEAT1 and alleviates neuronal damage

The results of RT-qPCR showed that miR-139 mimic cells were successfully constructed (Fig. 4A). After overexpression of miR-139, the activity of neurons was enhanced (Fig. 4B), cell apoptosis was reduced (Fig. 4C), inflammation level was reduced (Fig. 4D), and neuronal damage was relieved (Fig. 4E). At the same time, epileptic cells were treated with NEAT1 mimic and miR-139 mimic, which named NMM cells (Fig. 4F). Compared with epileptic cells treated with NEAT1 mimic (NM cells), NMM cells showed enhanced cell activity (Fig. 4G), reduced apoptosis (Fig. 4H), reduced inflammation level (Fig. 4I), and alleviated neuronal damage in epileptic rats (Fig. 4J).

ROCK1 accelerates neuronal damage in epileptic rats

The CTX-TNA2 and DI TNC1 cells were treated with si-ROCK1 or ROCK1-NC (Fig. 5A). Compared with ROCK1-NC treatment, si-ROCK1 treatment increased cell viability (Fig. 5B), decreased cell apoptosis (Fig. 5C), decreased inflammatory level (Fig. 5D), and decreased the neuronal damage (Fig. 5E).

The RhoA/ROCK signaling pathway promotes neuronal damage and epileptic formation

Western blot analysis detected the expression of the marker protein in the RhoA/ROCK signaling pathway. Compared with normal rats, epileptic rats showed higher protein expression of RhoA and Rock1. Compared with NEAT1 control treatment, NEAT1 mimic treatment increased the protein expression of RhoA and Rock1. Compared with the miR-139 control treatment, miR-139 mimic treatment decreased the protein expression of RhoA and Rock1 (Fig. 6).

Discussion

Epilepsy is a neurological disorder characterized by neuronal system dysfunction causing the incongruity of excitatory and inhibitory neurons in the CNS [17]. Our study uncovered a critical role of lncRNA NEAT1 on the pathological epilepsy progress. We found that NEAT1 exerted a pro-apoptotic effect by downregulating miR-139, thereby upregulating ROCK1 in the hippocampal neurons. These results for the first time implied an innovative pathway functioning to block epileptogenesis, which may signify a promising treatment for epilepsy.

It is well-admitted that ectopic manifestation of lncRNAs has been complicated in the epileptogenesis. For instance, a microarray analysis obtained that total of 105 lncRNAs in the hippocampus and 705 lncRNAs

in the cortex were remarkably dysregulated in the epilepsy model [18]. Prior researches have recommended that lncRNA NEAT1 is a critical regulator in malignancy progression, and higher NEAT1 expression is connected with cell metastasis and invasion [19–21]. LncRNA NEAT1 has also been validated to be connected with sepsis-induced myocardial cell injury and the cardiomyocyte apoptosis induced by H₂O₂ [22, 23]. Interestingly, a study also found that NEAT1 affected cell viability and inflammatory reaction in epilepsy cell model induced by IL-1 β [24]. Thus, we established rat and cell epilepsy models with the intention of measuring the expression form of lncRNA NEAT1. The current study presented that NEAT1 is upregulated in the SE-induced rats. We further suggested that overexpression of NEAT1 deteriorate the hippocampal damage.

Accumulating reports discovered that lncRNAs can work as ceRNA for miRNAs to competitively restrain miRNAs. For instance, lncRNA UCA1, which was downregulated in the epileptiform hippocampal neurons and rat of temporal lobe epilepsy, could obstruct seizure-induced brain injury by modulating miR-495 expression [25]. lncRNA H19 was upregulated during the progression of temporal lobe epilepsy and H19 overexpression provoked SE-induced neuronal apoptosis through sponging let-7b [26]. Based on our finding that overexpression of NEAT1 promoted the neuronal apoptosis, we further studied the functional target of NEAT1. Using microarray analysis, we uncovered that NEAT1 might target miR-139 directly and dual luciferase reporter gene assay validated the findings. It has been uncovered that miR-139 dysregulation was connected with onset and progression of assorted malignancies such as prostate [27], hepatocellular [28], and hepatocellular cancer [29]. Besides, miR-139 was also found to partake in the inhibitory function of monomethyl fumarate which exerted on oxidative stress and inflammatory response in intracerebral hemorrhage-induced rat brain damage [30]. Down-regulated miR-139-5p was found both in patients with temporal lobe epilepsy and rat model of SE, suggesting an innovative therapeutic target for epilepsy [31]. More importantly, a recent study validated the miR-139-5p overexpression could accelerate survival of neurons, block the apoptosis, and ameliorate neuronal damage in epilepsy [32]. In line with these aforementioned studies, we revealed that miR-139 was down-regulated in hippocampal tissues and neurons of the SE rats. NEAT1 could regulate miR-139 expression and miR-139 overexpression could eliminate the pro-apoptosis ability of NEAT1 during the SE. Our results support to confirm the involvement of miR-139 during SE and presented further proof about its endogenic alteration through lncRNA.

Subsequently, we verified that miR-139 regulated ROCK1 expression negatively in the rat SE neurons. We found that ROCK1 was significantly upregulated in the SE-induced hippocampal neurons. Our results were supported by a study performed by Yuki Fujita et al. with the findings that the RhoA/ROCK signaling pathway was activated by lesions to the adult CNS in animals to block axon growth and sprouting [33]. Evidence provided by Quarta Serena et al demonstrated that the RhoA/ROCK axis was involved in the outgrowth mediation of adult peripheral neurons [34]. The RhoA/ROCK signaling pathway was involved in different pathological conditions including epilepsy and inactivation of the RhoA/ROCK signaling pathway is an efficient approach to treat CNS disorders [35]. We validated that ROCK1 knockdown could overturn the inhibitory functions of miR-139 in neuronal apoptosis.

Conclusion

In summary, this study verified that NEAT1 upregulation is involved in SE-induced neuronal damage by regulation miR-139/ROCK1 axis so as to regulate cellular apoptosis and inflammation (Fig. 7). These findings are conducive to our understanding of the specific mechanisms of seizure-induced hippocampal neuronal damage and might present an innovative therapeutic target of epilepsy.

Abbreviations

lncRNA: long non-coding RNA; miR: microRNA; CNS: central nervous system; NEAT1: nuclear-enriched abundant transcript 1; SE: status epilepticus; ATCC: American Type Culture Collection; HE: Hematoxylin-eosin; RT-qPCR: reverse transcription quantitative polymerase chain reaction; EdU: 5-ethynyl-2'-deoxyuridine; PBS: phosphate buffer saline; OD: optical density; WT: wild type; MUT: mutant; ANOVA: one-way analysis of variance; TNF: tumor necrosis factor; IL: interleukin; ELISA: Enzyme-linked immunosorbent assay.

Declarations

Ethics approval and consent to participate

All the animal related experiments were permitted by the Ethics Committee of Liaocheng People's Hospital and accomplished according to the Chinese Animal Welfare Act.

Consent to publish

Not applicable.

Availability of data and materials

All the data generated or analyzed during this study are included in this published article. And the identifying/confidential patient data are not be shared.

Competing interests

The authors have no conflicts of interest to declare.

Funding

Not applicable.

Authors' contributions

XWL1, FH and XWL2 designed the study. SXT, WHW, XXX and DG implemented the tests. XWL1 analyzed the data and FH, HLM, and XWL2 interpreted the data. XWL1 wrote the first draft of the manuscript, FH

and XWL2 reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

LncRNA NEAT1 expression is abnormally high in the hippocampal tissues of epileptic rats. A, HE staining for the damage of hippocampal tissues. B, microarray analysis of differentially expressed lncRNA; C, the expression level of NEAT1 in hippocampal tissues of normal and epileptic rats detected by RT-qPCR (* $p < 0.05$ according to the unpaired t test). D, levels of TNF- α , IL-6, IL-1 β in hippocampal tissues of normal and epileptic rats detected by ELISA (* $p < 0.05$ according to the unpaired t test). All the experiments were carried out independently for three times to take the average value.

Figure 2

The influence of NEAT1 overexpression on neurons of epileptic rats. A, IL-1 β content in the CTX-TNA2 and DI TNC1 cells determined by ELISA. B, NEAT1 expression in the CTX-TNA2 and DI TNC1 cells treated with

NEAT1 mimic/control detected by RT-qPCR (*p < 0.05 according to the two-way ANOVA). C, cell viability of the CTX-TNA2 and DI TNC1 cells treated with NEAT1 mimic/control detected by EdU assay (*p < 0.05 according to the two-way ANOVA). D, cell apoptosis of the CTX-TNA2 and DI TNC1 cells treated with NEAT1 mimic/control detected by flow cytometry (*p < 0.05 according to the two-way ANOVA). E, levels of TNF-α and IL-6 in the CTX-TNA2 and DI TNC1 cells treated with NEAT1 mimic/control detected by ELISA (*p < 0.05 according to the two-way ANOVA). F, HE staining of hippocampal tissues of epileptic rats injected with NEAT1 mimic/control. All the experiments were carried out independently for three times to take the average value.

Figure 3

NEAT1 mediates ROCK1 through regulating miR-139. A, the binding site of NEAT1 and miR-139 predicted by StarBase and the related luciferase activity. B, miR-139 expression detected by RT-qPCR. C, the binding site of miR-139 and ROCK1 predicted by StarBase and the related luciferase activity. D, ROCK1 expression detected by RT-qPCR. *p < 0.05 according to the two-way ANOVA. All the experiments were carried out independently for three times to take the average value.

Figure 4

miR-139 upregulation alleviates neuronal damage induced by NEAT1 overexpression. A, miR-139 expression in the CTX-TNA2 and DI TNC1 cells treated with miR-139 mimic/control detected by RT-qPCR. B, cell viability of the CTX-TNA2 and DI TNC1 cells treated with miR-139 mimic/control detected by EdU assay. C, cell apoptosis of the CTX-TNA2 and DI TNC1 cells treated with miR-139 mimic/control detected by flow cytometry. D, levels of TNF- α and IL-6 in the CTX-TNA2 and DI TNC1 cells treated with miR-139 mimic/control detected by ELISA. E, hippocampal damage in the CTX-TNA2 and DI TNC1 cells treated with miR-139 mimic/control observed under HE staining. F, miR-139 expression in NMM and NM cells. G, cells viability of NMM and NM cells detected by EdU assay. H, cell apoptosis of NMM and NM cells detected by flow cytometry. I, levels of TNF- α and IL-6 in NMM and NM cells detected by ELISA. J, hippocampal damage in NMM and NM cells observed under HE staining. NMM cells, the CTX-TNA2 and DI TNC1 cells treated with NEAT1 mimic and miR-139 mimic; NM cells, the CTX-TNA2 and DI TNC1 cells treated with NEAT1 mimic. * $p < 0.05$ according to the two-way ANOVA. All the experiments were carried out independently for three times to take the average value.

Figure 5

ROCK1 silencing decreased the neuronal damage. A, ROCK1 expression in the CTX-TNA2 and DI TNC1 cells treated with si-ROCK1 or ROCK1-NC detected by RT-qPCR. B, cell viability in the CTX-TNA2 and DI TNC1 cells treated with si-ROCK1 or ROCK1-NC detected by EdU assay. C, cell apoptosis in the CTX-TNA2 and DI TNC1 cells treated with si-ROCK1 or ROCK1-NC detected by flow cytometry. D, levels of TNF-α and IL-6 in the CTX-TNA2 and DI TNC1 cells treated with si-ROCK1 or ROCK1-NC detected by ELISA. E, hippocampal damage in the CTX-TNA2 and DI TNC1 cells treated with si-ROCK1 or ROCK1-NC observed under HE staining. * $p < 0.05$ according to the two-way ANOVA. All the experiments were carried out independently for three times to take the average value.

Figure 6

The RhoA/ROCK signaling pathway was activated in epileptic cells. Protein expression of RhoA and ROCK1 in normal, epileptic cells, epileptic cells treated with NEAT1 control/mimic, and miR-139 control/mimic. * $p < 0.05$ according to the two-way ANOVA. All the experiments were carried out independently for three times to take the average value.

Figure 7

Molecular mechanism of NEAT1, miR-139, and ROCK1 in epileptic rats. LncRNA NEAT1 inhibited the expression of miR-139, promoted the expression of ROCK1 and the activity of RhoA/ROCK1 pathway, weakened the activity of astrocytes, enhanced apoptosis and increased the level of inflammatory factors, thus promoting neuronal damage and aggravating the epilepsy symptoms of rats.

Supplementary Files

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