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LncRNA HOTAIR Modulates Lipopolysaccharide-Induced Inflammatory Injury in Neuronal Cell Line HT-22

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ABSTRACT Epilepsy was recently emerged as a prevalent neurological disorder of the central nervous system (CNS). the In this work, the researchers aimed to explore the functional character of one of non-coding RNA (lncRNA) Hox transcript antisense RNA (HOTAIR) in hippocampal HT-22 after lipopolysaccharide (LPS) modulation. An in vitro inflammatory cell-based model was constructed by LPS treatment and inflammatory cell injury was monitored through the changes of cell viability, cell apoptosis, and levels of inflammatory cytokines. HOTAIR expression was inhibited via cell transfection, and the impact of HOTAIR depletion on LPS modulated cell inflammatory injury, and key kinases of NF-κB and MEK/ERK pathways were investigated after LPS stimulation by the qRT-PCR method. Results presented that LPS treatment led to the upregulation of HOTAIR in HT-22 cells, and LPS-induced cell inflammatory injury was reduced by HOTAIR knockdown. Intriguingly, HOTAIR depletion suppressed the phosphorylated levels of crucial kinases of both NF-κB and MEK/ERK pathways. Hence, this study concluded that LPS upregulated HOTAIR, and HOTAIR could modulate the LPS-induced cell inflammatory injury via NF-κB and MEK/ERK pathways.

INTRODUCTION

Epilepsy is marked by the natural recurrent seizures instigated by atypical transmission of the electrical signals from the central nervous system (CNS) (Fisher et al. 2014; Leach and Abassi 2013). Epilepsy, one of the most prevalent neurological disorder diseases that affects about 50 million people worldwide (Behr et al. 2016). The genetic syndrome, brain mass, brain damage, central nervous system tumours and infections are known currently as the risk factors for epilepsy (Singh and Trevick 2016). Although a majority of people with epilepsy experience remission via receiving either monotherapy or combination therapy with antiepileptic drugs, appropriately five to thirty-five percent of them still have seizures and are resistant to the drug (Gschwind and Seeck 2016). As a result, more effective treatment strategy is urgently needed to cure epilepsy. As reported, blood-brain barrier (BBB) plays an important part in maintaining brain homeostasis by regulating the solute flux (de Wit et al. 2017). Systemic inflammation could disrupt BBB and cause the dysfunction of BBB, which is regarded as a biomarker of epileptogenesis (Bar-Klein et al. 2017). A rising evidence attributes the precipitation and recurrence of epilepsy to the inflammation of cerebral parenchyma (Bozzi and Caleo 2016). Inflammation is considered to be an important cause of epilepsy, different from the conventional mechanisms that cause epilepsy, such as the imbalance of excitatory and inhibitory neurotransmitter systems, and the hormone disorders (Andrzejczak et al. 2016). Hence, the deep exploration of the protection of inflammatory signal interdiction in epileptogenesis and the response of neurons to inflammation was of great significance (Ji et al. 2018).

LncRNAs refer to the families of non-coding RNAs (ncRNAs) containing endogenous nucleotides with a length of greater than 200, and are emerging as the significant regulator in various human diseases (Prensner and Chinnaiyan 2011). Recently, lncRNAs are reported by copious literature to implicate actively in the pathogenesis

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of epilepsy (Lee et al. 2015; Geng et al. 2018; Jang et al. 2018; Wu and Yi 2018). LncRNA Hox transcript antisense RNA (HOTAIR) is largely found in the brain, and the multivariate analyses indicated that the relative expression level of HOTAIR was related independently to the grades of glioma (Xavier et al. 2018). ROC curve analysis demonstrated that the expression level of HOTAIR more than 0.40 represents the moderate value when applying to predict II-IV grades (Zhao et al. 2019). However, the HOTAIR-related mechanistic regulation in epilepsy remains elusive. Currently, lipopolysaccharide (LPS) was utilized to construct the in vitro inflammation cell model during epilepsy. The possible regulation of HOTAIR and the underlying mechanism were investigated in LPS-treated HT-22 cells.

Objectives

This work aimed to explore the functional characteristic of HOTAIR in hippocampal HT-22 after lipopolysaccharide (LPS) stimulation to see if modulated HOTAIR could play an affect NF-êB and MEK/ERK pathways during LPS-induced cell inflammatory injury.

METHODOLOGY

Cell Culture and Treatment

HT-22, the neuronal cell line of mouse hippocampal was procured from Procell Life Science and Technology (Wuhan, China). Cell sample of HT-22 was allowed to grow in the Dulbecco's modified Eagle's medium (DMEM) obtained from Gibco, Grand Island, NY, USA in a humidified incubator filled with five percent CO_2 and ninety-five percent air, at 37°C. Ten percent (v/v) foetal bovine serum (FBS; Gibco) and one percent (v/v) penicillin-streptomycin solution (Invitrogen, Carlsbad, CA, USA) served as the supplements for DMEM. For LPS treatment, HT-22 cell sample was cultivated in the DMEM adding the 1 ìg/ml of LPS (Sigma-Aldrich, St. Louis, MO, USA) for 16 hours.

Cell Transfection

For plasmid transfection, the triple HOTAIR-specific short hairpin RNAs (shRNAs), termed sh-HOTAIR-1, sh-HOTAIR-2 and sh-HOTAIR-3, were designed and produced by GenePharma Co., Ltd. (Shanghai, China), along with the shRNAs negative control (NC), termed sh-NC. These plasmids were severally transfected into HT-22 cell samples by application of Lipofectamine™ 3000 Transfection Reagent (Invitrogen). The targeting oligoribonucleotides of HOTAIR-specific shRNAs were as follows: sh-HOTAIR-1, 5'-GCCCCGTCGCGGAAGGTGTGGCGTCAAG-3'; sh-HOTAIR-2, 5'-ACTGGGGTCTCTACGAACCCAAAGACGA-3'; sh-HOTAIR-3, 5'-TCAATTATAGCGGGCGTTACCAACGCGA-3'; and sh-NC, 5'-CATGCAAACTGAGTATTCGCTGGATCGG-3'.

Cell Viability Detection

HT-22 cell samples were planted at the density of 1×10^4 cells/well in the 96-well plates. After LPS treatment, 10 μL of reagent (Cell Counting Kit-8 (CCK-8); Dojindo Laboratories, Kumamoto, Japan) was added into each well for 2 hours at 37°C. Cell viability was monitored by testing the absorbance at 450 nm.

Flow Cytometer for Cell Apoptosis

The cell apoptosis rate was monitored by applying double staining (Annexin V-FITC) and propidium iodide (PI). HT-22 cell samples in 6-well plates (1×10^5 cells/well) were treated with LPS, then collected and re-suspended in the Binding buffer. Following double staining as per the protocol of Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, San Jose, CA), the percentage of apoptotic cell samples were determined by flow cytometer (BD Biosciences).

Western Blot

The protein extracts from HT-22 cell samples were acquired by culturing in RIPA buffer, then quantitated and diluted in loading buffer to the same concentration. Following separation by electrophoresis on twelve percent SDS-PAGE gel, samples were shifted onto the polyvinylidene difluoride (PVDF) membrane and treated with the five percent bovine serum albumin (BSA; Beyotime, Shanghai, China). Samples were then probed with the diluted primary antibodies

against the internal reference GAPDH (ab8245; Abcam, Cambridge, MA) and Bax (ab32503), Bcl-2 (ab32124), p-p65 (ab86299), p65 (ab16502), IkBα (ab7217), p-IkBα (ab133462), MEK (ab32091), p-MEK (ab96379), ERK (ab184699) and p-ERK (ab201015) at 4°C all night. After washing in the Tris-buffered saline with 0.1 percent Tween-20 (TBST) membranes were probed with the horseradish peroxidase (HRP)-tagged secondary antibodies (Abcam) for 2 hours. Samples were finally immersed in the electrochemiluminescence (ECL) luminous liquid (Pierce, Rockford, IL).

Caspase-3 Activity Detection

The caspase-3 activity kit (Solarbio, Beijing, China) was commercially acquired and employed in line with the user manual. Cell samples of HT-22 in the 96-well plates were prepared for mixing with the reaction buffer and caspase-3 substrate at 37°C for 4 hours. The reaction mixture was assayed by a microplate reader at 405 nm.

Enzyme-linked Immunosorbent Assay (ELISA)

The culture supernatants were collected from the 24-well plates after treatment or transfection. Levels of tumour necrosis factor-α (TNF-α), interleukin (IL)-10, IL-6 and IL-β in culture supernatants were analysed by ELISA kits of R&D Systems (Minneapolis, MN) based on the supplier's instruction.

Real-time Polymerase Chain Reaction (qRT-PCR)

Based on the recommendations provided by the manufacturer, the total RNAs were removed from the cultured HT-22 cell samples with TRIzol reagent (Invitrogen) and complementary DNA (cDNA) was obtained from it using PrimeScript RT Reagent Kit (TaKaRa, Otsu, Japan). The expression level of HOTAIR was quantified by qPCR using SYBR Green PCR Master Mix (Invitrogen) and Real-Time PCR System (StepOne Plus, Applied Biosystems, Foster City, CA). HOTAIR expression was standardized to the internal reference GAPDH and calculated via the proportional delta-delta CT method ($2^{-\Delta\Delta Ct}$).

Statistical Analysis

Each assay was independently repeated in triplicate. Results are provided in form of the mean ± standard deviation (SD). Data analysis was processed by Prism (GraphPad, San Diego, CA). P-value was calculated via Student's t-test and one-way analysis of variance (ANOVA), with a significant level at less than 0.05.

RESULTS

Before considering the results, they were verified to check if the inflammatory HT-22 cell model was successfully established. Using the established model, researchers first assessed the cell viability and cell apoptosis using CCK-8 and flow cytometer assays, then measured the inflammatory cytokines using ELISA assay.

LPS Treatment Induced HT-22 Cell Injury and Released Inflammatory Cytokines

As shown in Figure 1A, the viability of HT-22 cells was decreased after treating with LPS ($^{**}p < 0.01$). However, under LPS treatment, the percentage of apoptotic HT-22 cells was significantly augmented, in comparison with the control group ($^{**}p < 0.01$; Fig. 1B). Likewise, data of western blot indicated that the level of anti-apoptosis protein Bcl-2 was weakened, while the level of pro-apoptotic protein Bax was enhanced in the LPS-group (Fig. 1C). The upregulated caspase-3 activity was also detected in LPS-group and presented in Figure 1D. The researchers then analysed the levels of IL-1β, IL-6, IL-10, and TNF-α after treating HT-22 cells with LPS. ELISA results showed that the released levels of various cytokines and TNF-α into the culture medium were all remarkably elevated after treating with LPS, comparing to the control group (all $^{**}p < 0.01$; Fig. 1E). All the above results suggested the successful establishment of a cell model with inflammatory injury via LPS stimulation.

HOTAIR Expression Upregulated in LPS-treated HT-22 Cells

The expression level of HOTAIR in LPS-treated HT-22 cells was analysed by qRT-PCR meth-

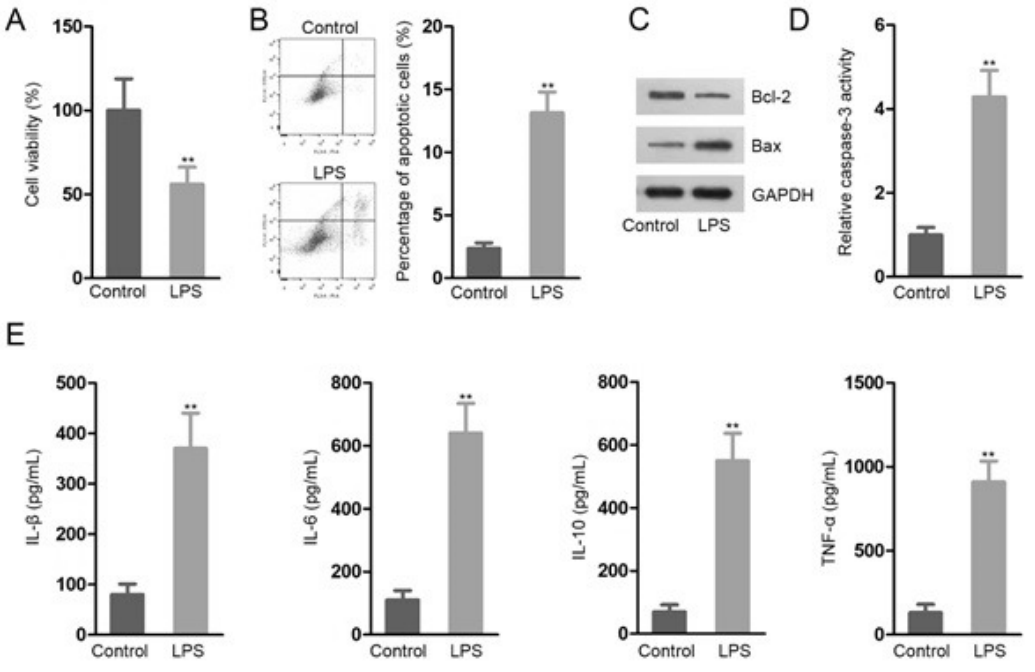

Fig. 1. LPS treatment induced HT-22 cell injury and released inflammatory cytokines. A. The cell viability of HT-22 detected by CCK-8 assay. B. The percentage of apoptotic HT-22 cells assayed by flow cytometer. C. Western blot analysis for the levels of apoptosis-associated proteins (Bcl-2 and Bax). D. Caspase-3 activity detection. E. Release of inflammatory cytokines in culture medium were estimated by ELISA assay. Non-treated cells served as the control. All data were exhibited as the mean ± SD. $^{**}p < 0.01$

od. Experimental result of Figure 2 presented that HOTAIR level was obviously upregulated in the LPS-group versus that in the control group ($^{**}p < 0.01$). Data showed that HOTAIR expression level was upregulated after treating HT-22 cells with LPS.

HOTAIR Depletion Ameliorated LPS-induced Inflammatory Injury of HT-22 Cells

Firstly, the researchers estimated the transfection effectiveness of HT-22 cells with the three specific shRNAs to HOTAIR using qRT-PCR. When linked to the sh-NC group, the expression level of HOTAIR in HT-22 cells transfected with sh-HOTAIR-1/2/3 was markedly increased, particularly in sh-HOTAIR-3 group ($^{**}p < 0.01$; Fig. 3A), showing that HOTAIR was expressed non-physiologically after specific transfection. Therefore, the researchers then implemented

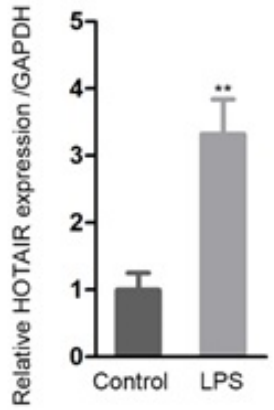

Fig. 2. HOTAIR expression was upregulated in LPS-treated HT-22 cells. Expression level of HOTAIR was analyzed using qRT-PCR after treating HT-22 cells with LPS. Non-treated cells served as the control. All data were exhibited as the mean ± SD. $^{**}p < 0.01$

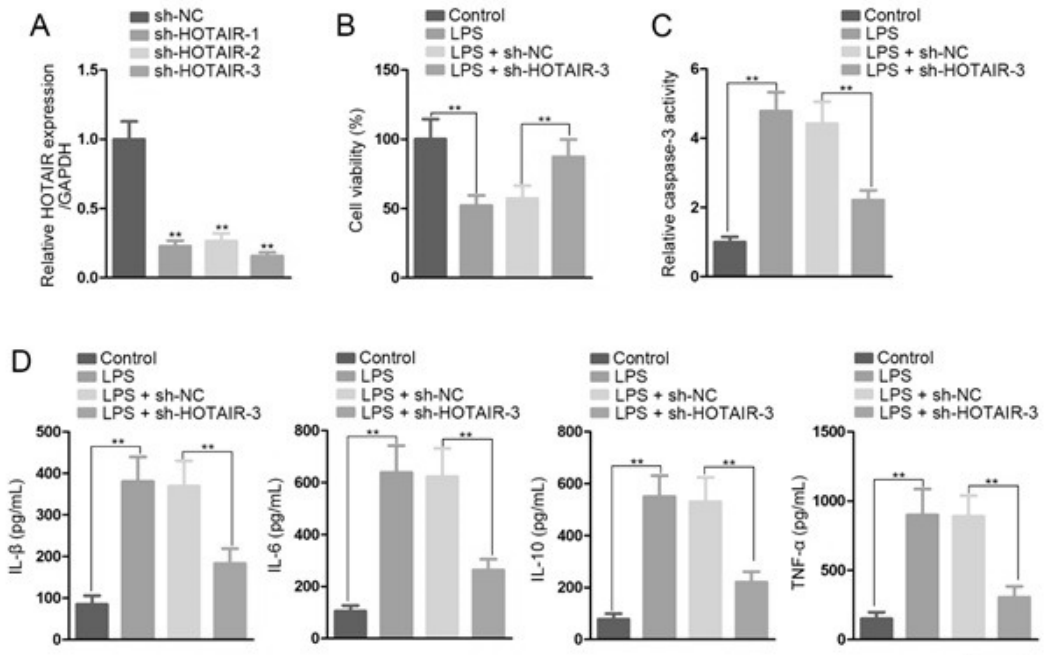

Fig. 3. HOTAIR depletion ameliorated LPS-induced inflammatory injury of HT-22 cells. A. HOTAIR expression level in HT-22 cells transfected with specific shRNAs to HOTAIR, versus sh-NC group. B. Cell viability by CCK-8 assay after inhibiting HOTAIR in LPS-treated HT-22 cells. C. Caspase-3 activity was assessed. D. ELISA assay was conducted for the levels of inflammatory cytokines in HT-22 cells responding to HOTAIR depletion. Non-treated cells served as the control. All data were exhibited as the mean ± SD. $^{**}p < 0.01$

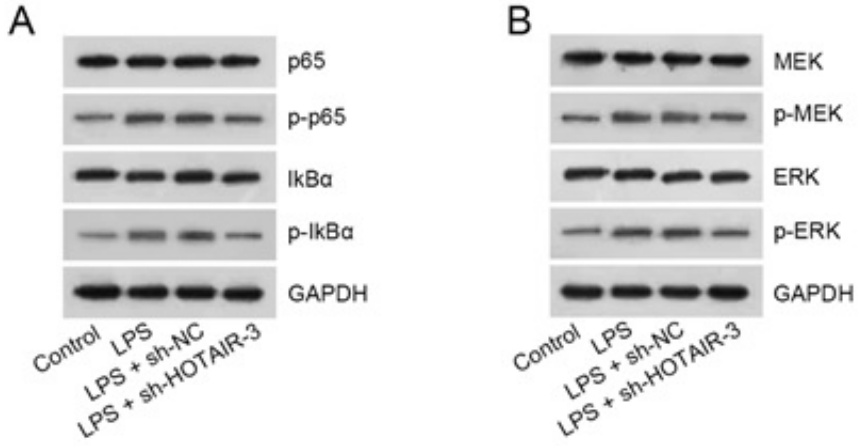

Fig. 4. HOTAIR depletion inhibited the LPS-induced activation of NF-κB and MEK/ERK pathways in HT-22 cells. A. The levels of critical kinases of NF-κB pathways was assayed by western blotting in control, LPS, LPS + sh-NC and LPS + sh-HOTAIR-3 groups. B. The levels of key kinases of MEK/ERK pathway in each group. Non-treated cells served as the control. All data were exhibited as the mean ± SD.

assays utilising the sh-HOTAIR-3. Subsequent CCK-8 assay demonstrated that the decrease of cell viability by LPS was reversed by HOTAIR depletion in comparison with the LPS + sh-NC group (both $^{**}p < 0.01$; Fig. 3B). The increased caspase-3 activity by LPS was reduced by silencing HOTAIR expression in HT-22 cells (both $^{**}p < 0.01$; Fig. 3C). Importantly, the expressions of these cytokines and TNF-α were upregulated by LPS, but lowered due to the HOTAIR knockdown when compared with the LPS + sh-NC group (all $^{**}p < 0.01$; Fig. 3D). Taken together, HOTAIR depletion attenuated the LPS-induced injury of HT-22 cells.

HOTAIR Depletion Inhibited the LPS-induced Activation of NF-kB and MEK/ERK Pathways in HT-22 Cells

To probe into the underlying HOTAIR-related mechanistic regulation, the phosphorylated levels of critical kinases of NF-κB and MEK/ERK pathways were assayed by western blotting. In Figures 4A and B, the researchers observed that the levels of phosphorylated p65, IêBá, MEK, and ERK were all elevated under LPS treatment. In addition, their upregulations induced by LPS were all weakened in response to the HOTAIR depletion. Data manifested that the activated NF-κB and MEK/ERK pathways by LPS could be inhibited by repressing HOTAIR expression in HT-22 cells.

DISCUSSION

Of the world's disease burden, epilepsy caused by atypical neuronal excitability occupies a considerable proportion, and the in-depth study of epilepsy has become a hot topic. In this study, HOTAIR was identified to be upregulated in HT-22 cells under LPS stimulation. Then, it was observed that HOTAIR depletion ameliorated the LPS-induced cell inflammatory injury. More importantly, MEK/ERK and the NFêB pathways activated by LPS treatment were suppressed by silencing HOTAIR expression. LPS is widely applied for establishing the in vivo or in vitro experimental inflammation model (Jamal Uddin et al. 2016; Jiang et al. 2016). Moreover, the multiple studies concerning the mechanism that contributed to epilepsy development and the potential treatments for epilepsy are implemented utilising the murine hippocampal HT-22 cells (Rahn et al. 2014; Zheng et al. 2015). Accordingly, the researchers first constructed the in vitro inflammatory HT-22 cell model using LPS stimulation. Cell viability, cell apoptosis and released cytokines were all assayed for verifying whether cell model was successfully established. The endotoxin LPS could decrease proliferative cells and increase apoptotic cells in various cell types (Guo et al. 2014; Liu et al. 2017). Likewise, proliferative HT-22 cells were markedly reduced, but apoptotic cells were induced by LPS. During cell apoptosis, the Bcl-2 family plays the key role in modulating programmed cell death (Ashkenazi et al. 2017). The researchers found that anti-apoptotic protein Bcl-2 was reduced but pro-apoptotic protein Bax was increased, following the obvious upregulation of caspase-3 activity. Thus, LPS induced cell injury in mitochondrial- and caspase-dependent ways. More intriguingly, levels of pro-inflammatory cytokines were distinctly enhanced, showing that HT-22 cells was inflamed significantly.

As mentioned above, HOTAIR was reported to implicate in the progression of glioma (brain disease), identified as the independent risk factor for II-IV grades. The researchers therefore hypothesised that HOTAIR might be implicated in the development of epilepsy. The HOTAIR expression level in HT-22 cells with inflammatory injury was then analysed. qRT-PCR data presented the upregulation of HOTAIR expression under LPS treatment, which was consistent with the study of Obaid et al. reporting that HOTAIR was overexpressed in LPS-treated macrophages (Obaid et al. 2018). Similarly, Zhang et al. reported that HOTAIR expression was induced by LPS in human hepatocellular carcinoma cell lines (Zhang et al. 2019b). Subsequently, HT-22 cells stimulated with LPS were transfected with HOTAIR silencing plasmids, followed by detection of cell proliferation, cell apoptosis, and levels of pro-inflammatory cytokines. Early studies have uncovered that HOTAIR could affect the apoptosis of cardiomyocytes, breast cancer cells, nucleus pulposus cells through affecting apoptotic related protein Bax, Bcl-2, and caspase-3 (Zhang et al. 2019a; Li et al. 2019; Yu et al. 2018). In the present study, LPS-induced inflammatory cell injury was attenuated by HOTAIR deple-

tion. To the researchers' knowledge, this report is the first to reveal the role of HOTAIR in LPS-treated HT-22 cells. During LPS-induced inflammation, the ligation of toll-like receptor 4 and LPS causes the activation of NF-κB and MAPK pathways, as well as the inflammatory mediators (Yoo et al. 2012). The researchers thus further analysed these signalling pathways in HT-22 cells abnormally expressing HOTAIR.

CONCLUSION

HOTAIR expression was strengthened in HT-22 cells by LPS. The depletion of HOTAIR weakened the LPS-induced cell inflammatory injury via NF-κB and MEK/ERK pathways. Data showed that the activation of these two signaling cascades induced by LPS was strongly hindered by HOTAIR depletion, indicating that the regulation of HOTAIR was linked with NF-κB and MEK/ERK pathways.

RECOMMENDATIONS

This study might provide a hypothetical source for the in-depth study of neuronal inflammation-induced epilepsy. However, more investigations should be implemented in vivo to verify the detailed role of HOTAIR.

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