The mmu_circ_0000335 inhibits M1 microglial-induced hippocampal neuronal apoptosis via the miR-19b-3p/SOCS1 axis in a mouse epilepsy model

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Abstract

Background: Increasing evidence has demonstrated that circular RNAs (circRNAs) participate in epileptogenesis, but the expression profile and role of circRNAs in epilepsy remain unknown. A circRNA microarray was performed to examine epilepsy-related circRNAs. Bioinformatics analyses, luciferin reporter experiments and real-time quantitative PCR (Rt-qPCR) in vitro experiments were performed to demonstrate the mechanism of circRNA-mediated gene regulation of the microglial phenotype under epileptic conditions. Then, to further confirm the effect of circRNAs on nerve damage in the hippocampus, a mouse model of epilepsy was established by intraperitoneal injection of lithium chloride and pilocarpine.

Results: The data indicated that 364 circRNAs were differentially expressed comparing epilepsy and control tissues. In particular, mmu_circ_0000335 expression was significantly downregulated in epileptic mice which was confirmed by Rt-qPCR. Overexpression of mmu_circ_0000335 promoted BV2 cell transformation into the M2 macrophage phenotype by increasing expression of CD206, Arg1, Ym1 and IL-10 while decreasing M1 macrophage markers IL-1β, IL-6, TNF-α and IFN-γ expressions under epileptic conditions. mmu_circ_0000335 expression triggered upregulation of Suppressor of Cytokine Signaling 1 (SOCS1) by decreasing miR-19b-3p levels, as determined by luciferase reporter assay. In vivo studies found that mmu_circ_0000335 overexpression decreased epilepsy-induced neural cell apoptosis in the hippocampus by reducing inflammatory cytokine expression. Immunofluorescence detection showed that mmu_circ_0000335 overexpression promoted microglial transformation into the M2 phenotype which had an anti-inflammatory effect.

Conclusions: These results collectively indicated that mmu_circ_0000335 was involved in epilepsy progression by functioning as a miR-19b-3p sponge to enhance SOCS1 expression. Thus, mmu_circ_0000335 may be a candidate therapeutic target for
Background

Epilepsy is a chronic brain disorder identified by recurrent seizures presence. Among the 70 million people estimated to suffer epilepsy, 60 million live in medium- or low-income countries, around half of which are fairly young population [1]. Impaired regulation of the activation and resolution of inflammatory molecules and cells in injured neuronal tissue is an essential factor in the development of epilepsy [2]. The genes involved in epileptogenesis are highly regulated by a variety of epigenetic regulatory mechanisms, and their chronic alteration in the hippocampus may function significantly in temporal lobe epilepsy [3–5]. Amongst them, micro RNAs (miRNAs), which are small (20–24 bp) noncoding RNAs (ncRNAs) that regulate hundreds of target genes, have been deeply studied [6, 7]. Nevertheless, recent research has illustrated that gene expression is more accurately modified by another ncRNA, which is called circular RNA (circRNA) [8–10], but the regulatory mechanisms are unclear.

The circRNAs are a circular-shaped and covalently closed ncRNAs subgroup [8, 9] most often produced by back-splicing, which is a procedure that downstream exons are reversely spliced onto upstream exons by. The circRNAs have now become known as the main epigenetic regulators in various disease pathogenesis [11–13]. A circRNA may interact with the gene transcription machinery to produce the corresponding linear form of the mRNA [14]. Furthermore, circRNAs include miRNA binding sites called miRNA response elements that enable circRNAs to sequester the target miRNA, an effect that has been described as “miRNA sponge effect” [8, 9, 15]. From this perspective, circRNAs can modify their target gene expression through regulatory networks of circRNA-miRNA-mRNA.

In present investigation, we validated the comprehensive profile of differentially regulated circRNAs in the mouse hippocampus pilocarpine-induced epilepsy model and identified
their mechanisms in the epigenetic regulatory processes involved in chronic epilepsy pathophysiology.

Materials & Methods

Ethics statement

We purchased male C57BL/6 mice aged six to eight weeks from the SHANGHAI SLAC LABORATORY ANIMAL CO. LTD. (Shanghai, China). We housed the mice under controlled room temperature (22 °C) and photoperiods (12 h light/12 h dark) in a specific pathogen-free conditioned animal care facility. Ethics Committee of the Hunan Brain Hospital, Clinical Medical School approved the study (HBH20150628).

Epileptic mouse model

We injected lithium chloride (125 mg/kg, intraperitoneal injection; Sigma-Aldrich, St. Louis, MO, USA) 20 hours prior to pilocarpine administration (50 mg/kg, intraperitoneal injection; Sigma-Aldrich) to induce epileptic seizures. Pretreatment with atropine (1 mg/kg, intraperitoneal injection; Sigma-Aldrich) 0.5 h prior to pilocarpine administration was employed to repress peripheral cholinergic effects. Racine’s scale was utilized to validate the convulsion severity. Epilepsy (identified with a Racine’s scale score of 4 or 5) was defined as the beginning of a set of continuous generalized seizure activity lasting at least 40 min. Seizure latency was also recorded. Epilepsy was terminated by diazepam injection (10 mg/kg, intraperitoneal injection; Hospira) 3 h after epileptic induction. We injected control mice intraperitoneally with an equal volume of saline instead of pilocarpine. To study the effect of mmu_circ_0000335 on epileptic-induced hippocampal neuronal injury, an adeno-associated viral (AAV) plasmid carrying mmu_circ_0000335 was synthesized and purchased from GeneChem Co., Ltd. (Shanghai, China) and mmu_circ_0000335 overexpression was induced by stereotactic injection in the hippocampus with the AAV-mediated intervention system as previously reported [16, 17].
Mice were euthanatized every 14 days after SE was induced. Prior to euthanasia, the rats were anesthetized with 3% pentobarbital sodium (Merck) and subsequently perfused with saline. Hippocampal tissues were dissected from the animals and frozen immediately.

Expression profile analysis of circRNAs

Brain samples from epileptic and normal mice were utilized for circRNA microarray. Tissues specimens were taken during operation and immediately frozen at –80°C for further use. The circRNA chip (ArrayStar Human circRNAs chip; ArrayStar, Rockville, MD, USA) containing 5,639 probes specific for mice circRNA splicing sites was used. Following hybridization and washing of the samples, one pair of epileptic and control samples were analyzed on the circRNAs chip. Exogenous RNAs developed by the External RNA Controls Consortium (ERCC) were employed as controls. circRNAs were enriched by digesting linear RNA with RNase R (Epicentre, Madison, WI, USA). Labeled RNAs were scanned by an Agilent Scanner G2505C (Agilent Technologies, Santa Clara, CA, USA). The circRNA microarray process was performed by KangChen Biotech, Inc. (Shanghai, China).

Bioinformatics analyses

We used the bioinformatics analysis website Circular RNA Interactome for circRNA and miRNA interaction predictions (https://circinteractome.nia.nih.gov/miRNA_Target_Sites/mirna_target_sites.html). The target site between miR–19b–3p and the 3’-UTR of SOCS1 was predicted using the TargetScan web-based tool (http://www.targetscan.org/vert_71/).

Luciferase reporter assay

We amplified and cloned fragments of Suppressor of Cytokine Signaling 1 (SOCS1) and the mmu_circ_0000335 3’-untranslated region (UTR) including miR-19b-3p binding sites into the psiCheck2 reporter vector (Promega, Shanghai, China). We co-transfected 293T cells with the reporter vector and miR-19b-3p mimics or negative control sequence (miR-NC).
using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 h, we harvested the cells and performed dual-luciferase reporter assays following the procedure specified for the Dual-Luciferase Reporter Assay System (Promega). The data were expressed as the ratio of experimental (Renilla) luciferase to control (Firefly) luciferase.

*Rt-qPCR*

We extracted total RNA using TRIzol (Invitrogen) following the manufacturer’s protocol. We then performed reverse transcription with the One Step SYBR PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd.). We performed the reaction using the ABI PRISM 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA) at 42°C for 5 min, 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec, 55°C for 30 sec and 72°C for 30 sec. We conducted three independent experiments for every sample. We analyzed data by comparing the $2^{-\Delta\Delta Ct}$ value. GAPDH and U6 were used as internal controls.

*Immunohistochemistry and immunofluorescence assay*

Hippocampal samples were fixed in 10% formalin and embedded in paraffin. We stained 5-μm sections with TUNEL stain for apoptosis analysis and stained nuclei using DAPI. We prepared for staining the cells or tissues with Iba-1 and CD206 antibodies at 4°C overnight, then incubated them with conjugated secondary antibody for 1 h at room temperature in the dark. After washing the slides several times with phosphate-buffered saline, we incubated them in DAPI for 3 min and mounted them in glycerol. We assessed fluorescence under a fluorescence microscope.

*Enzyme-linked immunosorbent assay*

We assayed interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) expressions using enzyme linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, UK) following the manufacturer’s protocol.
Cell culture and transfection of BV2 cells

We purchased BV2 cells from Wuhan Biofavor Biotechnology Service Co. Ltd., China and cultured them in 1640 Medium (1640; Invitrogen) with fetal bovine serum of 10% (Invitrogen) in a 5% CO\textsubscript{2} incubator. In order to mimic the epilepsy conditions, on in vitro day 6 we replaced the culture medium for 3 h with magnesium-free (Mg\textsuperscript{2+}-free) physiological solution (pH = 7.3) supplied with 145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 2 mM CaCl\textsubscript{2}, 0.002 mM glycine and 10 mM glucose. After treatment, we transferred the cells to conventional 1640 medium and maintained them as previously described.

To detect the mmu_circ_0000335, miR-19b-3p and SOCS1 effects on the epilepsy-induced microglial phenotype transition, we purchased an mmu_circ_0000335 overexpression vector, miR-19b-3p mimic, miR-19b-3p inhibitor and SOCS1 overexpression vector from GeneCopoeia (Shanghai, China) and transfected them into the cell line of BV2 with Lipofectamine 2000 transfection reagent (Invitrogen) following the standard protocol. We used the cells for the experiments after 48 h.

Flow cytometry analysis of apoptosis

BV2 cells from different groups were obtained, centrifuged, washed twice using PBS and resuspended in 1×Binding Buffer. We then added 5 \mu L Annexin V-FITC solution and 5 \mu L propidium iodide solution following the instructions for the Annexin V-FITC Apoptosis Detection Kit, BD Biosciences. We incubated cells in dark for 15 minutes under the room temperature. Thereafter, we utilized flow cytometry to define the viable apoptotic cell percentile.

Statistics analysis

Data are denoted by means ± standard deviation (SD). GraphPad Prism software (version 5.0) was employed to compare the differences among groups. A P value ≤ 0.05 was
accepted as significantly statistical difference.

Results

*The mmu_circ_0000335 expression in cerebral tissue was decreased after epilepsy*

Our aim was to determine the particular circRNAs that were differentially expressed comparing epileptic with control mice cerebral tissue. The circRNA microarray assay found that 364 circRNAs in total were differentially expressed by >3 fold in epileptic compared to control tissues. We then narrowed the detection to the 26 most aberrantly expressed circRNAs, which provided 15 upregulated circRNAs and 13 downregulated circRNAs, which were indicated in Figure 1A. We chose the 13 most dysregulated circRNAs for validation by Rt-qPCR in 10 sample pairs. The Rt-qPCR results showed significantly reduced expression of mmu_circ_0000335 in epileptic mouse cerebral tissue compared with controls (Figure 1B).

To determine if mmu_circ_0000335 played a role in the epilepsy-induced microglia phenotype, BV2 cells were induced under epileptic conditions. The results showed that epilepsy induced microglia into expressing M1 phenotypic markers IFN-γ, IL-6, TNF-α and IL-1β. In contrast, mmu_circ_0000335 overexpression suppressed the expression of these epilepsy-induced M1 phenotypic markers. mmu_circ_0000335 overexpression also promoted M2 phenotypic markers CD206, Arg1, Ym1 and IL-10 expressions (Figure 1 C-J), suggesting that mmu_circ_0000335 overexpression had a protective effect by promoting microglia into the M2 phenotype.

*The miR-19b-3p and SOCS1 were the downstream targets of mmu_circ_0000335.*

Bioinformatics analysis found that miR-19b-3p and SOCS1 were the downstream targets of mmu_circ_0000335. In order to identify the interactive relationship among mmu_circ_0000335, miR-19b-3p and SOCS1, a luciferase reporter vector was constructed and used to show that miR-19b-3p was a downstream binding target of
mmu_circ_0000335 (Figure 2A). Notably, mmu_circ_0000335 inhibited luciferase activity in wild-type cell lines but not in mutated cells (Figure 2B). To determine if SOCS1 was a potential miR-19b-3p target, bioinformatics analyses were conducted, which confirmed direct interactions of miR-19b-3p with the SOCS1 3’-UTR and subsequent suppression of SOCS1 mRNA (Figure 2C). In the luciferase reporter assay, miR-19b-3p inhibited the luciferase activity in wild-type cell lines, but not in mutated cells (Figure 2D), suggesting that mmu_circ_0000335 overexpression inhibited transformation of epilepsy-induced microglia into the M1 phenotype by targeting the miR-19b-3p/SOCS1 axis. The interaction among mmu_circ_0000335, miR-19b-3p and SOCS1 functions importantly in the phenotypic microglia switch under epileptic condition.

To further identify the effect of the mmu_circ_0000335, miR-19b-3p and SOCS1 interactions on the microglial phenotype, we transfected BV2 cells with mmu_circ_0000335, miR-19b-3p and SOCS1 overexpression vectors singly or combined. Rt-qPCR detection demonstrated that the mmu_circ_0000335 expression was increased significantly after transfected with the mmu_circ_0000335 overexpression vector; however, miR-19b-3p or SOCS1 overexpression had no effect on mmu_circ_0000335 expression, which suggested that mmu_circ_0000335 was upstream of both miR-19b-3p and SOCS1 (Figure 3A). Rt-qPCR assays also showed that mmu_circ_0000335 overexpression suppressed miR-19b-3p expression. miR-19b-3p mimics transfection promoted miR-19b-3p expression, but transfection with the SOCS1 overexpression vector had no effect on miR-19b-3p expression (Figure 3B), indicating that miR-19b-3p was downstream of mmu_circ_0000335, but upstream of SOCS1. Further study found that mmu_circ_0000335 overexpression promoted SOCS1 upregulation, while upregulation of miR-19b-3p suppressed SOCS1 expression. Overexpression of SOCS1 significantly enhanced SOCS1 expression, but had no effect on either mmu_circ_0000335 or miR-19b-
3p expression (Figure 3C). Therefore, we concluded that SOCS1 was the downstream of both mmu_circ_0000335 and miR-19b-3p.

Rt-qPCR analysis also found that mmu_circ_0000335 or SOCS1 overexpression pushed microglia into the M2 macrophage phenotype by increasing CD206, Arg1, Ym1 and IL-10 expression. miR-19b-3p overexpression suppressed M2 macrophage phenotypic protein expression and promoted microglia into the M1 macrophage phenotype by increasing IL-6, IL-1β, IFN-γ and TNF-α expression. SOCS1 overexpression reversed the M1 phenotype promotional effect of miR-19b-3p (Figure 3D-K), indicating that mmu_circ_0000335 overexpression promoted the microglial M1 phenotype by increasing SOCS1 expression and adsorption of miR-19b-3p under epileptic condition.

*The mmu_circ_0000335 overexpression decreased epilepsy-induced and microglial-mediated inflammatory factor expression and hippocampal neuron damage.*

To further show that mmu_circ_0000335 had a protective effect against epilepsy-induced hippocampal neuronal damage, an AAV plasmid incorporating mmu_circ_0000335 was synthesized and used to overexpress mmu_circ_0000335 by hippocampus stereotactic injection with the intervention system that mediated by AAV. The results showed that apoptosis of hippocampal neurons induced by epilepsy was downregulated by mmu_circ_0000335 overexpression (Figure 4A and 4B). ELISA found that inflammatory factor TNF-α, IL-1β and IL-6 expressions in cerebral tissue were increased in the epilepsy mouse model while mmu_circ_0000335 overexpression suppressed inflammatory factors TNF-α, IL-6 and IL-1β (Figure 4C-E). Meanwhile, immunofluorescence assays found that epilepsy induced microglial activation by promoting Iba-I expression. mmu_circ_0000335 overexpression did not decrease microglial activation, but instead promoted microglia into the M2 macrophage phenotype by inducing CD206 expression (Figure 4F-I), suggesting that mmu_circ_0000335 overexpression association with decreased epilepsy-induced and
M1 microglial-mediated inflammatory factor expression and hippocampal neuronal damage by promoting microglia into the M2 macrophage phenotype.

Discussion

Microglia are indispensable in secretion of chemical mediators, which promote inflammatory responses of the central nervous system. Increasing evidence has shown that activated microglia could secret proinflammatory cytokines as well as reduce the seizure threshold. In this manner, microglia might help generate seizures via releasing and responding to endogenous inflammatory mediators including IL–1β, TNF-α and IL-6 [18, 19]. While it is unclear how epilepsy augments inflammasome activation, in this study we found abnormal circRNA expression relative to the microglial phenotype. For example, mmu_circ_0000335 expression in epileptic mice was downregulated. Overexpression of mmu_circ_0000335 suppressed microglial activation induced by epilepsy and transformed microglia into the M1 phenotype. Microglia belong to immune effector cells that located in central nervous system and function in the initiation, development and prognosis of immune inflammatory reactions in the brain [20]. Microglia could be activated into extreme pro- or anti-inflammatory states in polarizing manners, which are defined as M1 and M2 phenotypes, respectively [21, 22]. The M1 phenotype correlates with the transcriptome of proinflammatory cytokines such as IL–1β, IL-6, IFN-γ and TNF-α. The M2 phenotype correlates with expression of anti-inflammatory cytokines including CD206, Arg1, Ym1 and IL-10. The classical M1 phenotype is associated with neurodegeneration, but the M2 phenotype is implicated in promotion and neuroprotection of neuronal tissue regeneration [23, 24]. In this study, we found that epilepsy could modulate microglia/macrophages toward M1 polarization, while mmu_circ_0000335 overexpression pushed microglia/macrophages toward M2 polarization. Our study further suggested that miR–19b–3p and SOCS1 were mmu_circ_0000335
downstream targets. The mmu_circ_0000335 overexpression promoted SOCS1 expression by sponging miR-19b-3p. SOCS1 belongs to the SOCS family, which contains a STAT-binding site within the promoter region that located at 16p13 [25]. SOCS1 is expressed in numerous tissues and a number of cytokines regulate the expression [26]. Previous studies have found that SOCS1 has a suppressive effect on inflammation [27, 28]. The current investigation found that mmu_circ_0000335 promoted SOCS1 expression which altered the microglia/macrophage balance towards M2 polarization under epileptic conditions. In this study, luciferase reporter experiments showed that SOCS1 was a miR-19b-3p target and that miR-19b-3p could sponge mmu_circ_0000335. In vivo studies also confirmed that mmu_circ_0000335 overexpression decreased epilepsy-induced and microglial-mediated inflammatory factor expression and reduced hippocampal neuronal damage by pushing the microglia/macrophage balance toward M2 polarization under epileptic conditions.

Conclusion

In conclusion, we have shown that mmu_circ_0000335 inhibited M1 microglial-induced hippocampal neuronal apoptosis by regulating the axis of miR-19b-3p/SOCS1 in a mouse epilepsy model.

Abbreviations

Rt-qPCR: real-time quantitative PCR; circRNA: scircular RNAs; SOCS1: Suppressor of Cytokine Signaling 1; miRNAs: micro RNAs; ncRNAs: noncoding RNAs; AAV: adeno-associated viral; 3’-UTR: 3’-untranslated region; IL-1β: interleukin-1β, IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; ELISA Kits: enzyme linked immunosorbent assay kits.

Declarations

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Authors’ contributions

LL (superliliang@126.com), QL (lixin2002lixin@sina.com), and PY (pingyang198476@163.com) designed the study. YZ (29519693@qq.com), WPK (1281117923@qq.com), QW (wangqin2006515@sina.com) and conducted the experiments. WP (497181684@qq.com) took part in study design and data collection. SSX (422261174@qq.com) and JL (53558059@qq.com) performed statistical analysis. ZY (29519693@qq.com) wrote the manuscript and PY (pingyang198476@163.com) revised the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by the local Animal Research Committee of the Hunan Brain Hospital, Clinical Medical School approved the study (HBH20150628).

Consent for publication
Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Differential expression of circRNAs in epilepsy and control samples of mouse cerebral tissue. (A) Based on the circRNA microarray results, the top 15 upregulated and top 15 downregulated circRNAs in epilepsy compared with control samples are shown in the heatmap. The red color represents high expression, whereas the green color represents low expression. (B) RT-qPCR verification of the expression of mmu_circ_0000335 in cerebral tissue of ten mice with epilepsy compared with controls. ***p<0.001 versus control group. (C–J) RT-
qPCR detection found that mmu_circ_0000335 overexpression promoted the transformation of BV2 cells into the M2 phenotype by increasing expression of CD206 (C), Arg1 (D), Ym1 (E) and IL-10 (F) and decreasing expression of M1 macrophage markers IL-1β (G), IL-6 (H), TNF-α (I) and IFN-γ (J) under epileptic conditions. Data are presented as means ± SD. ***p<0.001 versus epilepsy group.
miR-19b-3p and SOCS1 were the downstream targets of mmu_circ_0000335. (A) Binding sites of miR-19b-3p in mmu_circ_0000335 were predicted. The mutated version of the mmu_circ_0000335 is also shown. (B) Relative luciferase activity was determined 48 h after transfection with miR-19b-3p mimic/NC or with wild type/mutated mmu_circ_0000335 in HEK293T cells. Data are presented as means ± SD. ***P<0.001 versus control group. (C) Binding sites of miR-19b-3p in the 3'UTR of SOCS1 were predicted. The mutated version of the SOCS1 3'UTR is also shown. (D) Relative luciferase activity was determined 48 h after transfection with miR-19b-3p mimic/NC or with the wild type/mutated SOCS1 3'UTR in HEK293T cells. Data are presented as means ± SD. **P<0.01.
The interaction among mmu_circ_0000335, miR-19b-3p and SOCS1 plays an important role in the phenotype switch of microglia under epileptic conditions. (A–C) Rt-qPCR detection showed the expression of mmu_circ_0000335 (A), miR-19b-3p (B) and SOCS1 (C) in BV2 cells after different treatments. Data are presented as means ± SD. ***P<0.001 versus control group. ###P<0.001 versus mmu_circ_0000335 overexpression group. (D–K) Rt-qPCR detection showed the expression of M2 macrophage phenotypic markers CD206 (D), Arg1 (E), Ym1 (F) and IL-10 (G), and M1 macrophage phenotypic markers IL-1β (H), IL-6 (I), TNF-α (J) and IFN-γ (K) under epileptic conditions. Data are presented as means ± SD. ***P<0.001 versus control group. ###P<0.001 versus mmu_circ_0000335 overexpression group.
The mmu_circ_0000335 overexpression decreased epilepsy-induced and microglial-mediated inflammatory factor expression and hippocampal neuronal damage. (A and B) Immunohistochemical detection showing apoptosis of hippocampal neurons. Data are presented as means ± SEM (n=10). ***P<0.001 vs control; ###P<0.001 vs epilepsy group. Bars, 50 μm. (C–E) ELISA showed the expression of inflammatory factors TNF-α (C), IL-1β (D) and IL-6 (E) in cerebral
tissue. Data are presented as means ± SEM (n=10). ***P<0.001 vs control; ###P<0.001 vs epilepsy group. (F-I) Representative images showing the microglial phenotype. Data are presented as means ± SEM (n = 6). ***P<0.001 vs control; ###P<0.001 vs epilepsy group. Bars, 50 μm.

Supplementary Files

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