Rho-associated Protein Kinase 2 Confers Epileptogenesis through the Activation of Astroglial Stat3 Pathway

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Abstract

Patients with temporal lobe epilepsy (TLE) are prone to tolerance to antiepileptic drugs. Based on the perspective of molecular targets for drug resistance, it is necessary to explore effective drug resistant genes and signaling pathways for the treatment of TLE. We performed gene expression profiles in hippocampus of patients with drug-resistant TLE and identified ROCK2 as one of the 20 most significantly increased genes in hippocampus. In vitro and in vivo experiments were performed to identify the potential role of ROCK2 in epileptogenesis. In addition, the activity of Stat3 pathway was tested in hippocampal tissues and primary cultured astrocytes. The expression levels of ROCK2 in the hippocampus of TLE patients were significantly increased compared with the control group, which was due to the hypomethylation of ROCK2 promoter. Fasudil, a specific Rho-kinase inhibitor, alleviated epileptic seizures in the pilocarpine rat model of TLE. Furthermore, ROCK2 activated the Stat3 pathway in pilocarpine-treated epilepsy rats, and the spearman correlation method confirmed that ROCK2 is associated with Stat3 activation in TLE patients. In addition, ROCK2 was predominantly expressed in astrocytes during epileptogenesis, and induced epileptogenesis by activating astrocyte cell cycle progression via Stat3 pathway. The overexpressed ROCK2 plays an important role in the pathogenesis of drug-resistant epilepsy. ROCK2 accelerates astrocytes cell cycle progression via the activation of Stat3 pathway likely provides the key to explaining the process of epileptogenesis.

Introduction

The Rho GTPases belong to the Ras superfamily of 20 to 30 kDa GTP-binding proteins that have been involved in the regulation of a broad spectrum of cellular functions ¹. Rho activation inhibits neurite outgrowth and promotes growth cone collapse and axon retraction ². RhoA is a member of the Rho GTPases and is abundantly expressed in cells of the CNS ³. Recent research indicated that kainic acid-induced seizures result in bilateral RhoA activation in the cortex and hippocampus ⁴.

ROCK, one of the most studied downstream effectors of RhoA, is a serine/threonine kinase of 160 kDa that exists in two isoforms in mammals: ROCK1 and ROCK2 ⁵. Both ROCK1 and ROCK2 are widely expressed in human, rat and mouse tissues ⁶⁻⁸. ROCK1 is more prominent in liver, testes and kidney, whereas ROCK2 is more pronounced in brain ⁵. Y-27632 and Fasudil are known as specific Rho-kinase inhibitors and are clinically used to treat cerebral vasospasm following subarachnoid hemorrhage ⁹.

ROCK2 immunoreactivity was observed in the pyramidal neurons of the cerebral cortex and hippocampus and in the Purkinje cells of the cerebellum ¹⁰. ROCK activation is necessary for neurite retraction in the injured CNS ¹¹. Additionally, ROCK acts to reduce the degree of plasticity at hippocampal synapses during long-term potentiation ¹². Rho/ROCK pathway plays an important role in hippocampus-dependent long-term spatial memory ¹³, and is even considered as a potential drug target in various neurological disorders ¹⁴.

Recently, it has been reported that Rho/ROCK pathway plays a role in epilepsy induced by PTZ or MES ¹⁵, and directly involved in seizure-induced cell death *in vivo* and *in vitro* ¹⁶. In this study, we aimed to determine the expression and cellular distribution of ROCK2 in epilepsy and investigate the efficacy of Fasudil in lithium-pilocarpine induced rat model of epilepsy. Furthermore, we demonstrated that ROCK2 induces gliosis via activating Stat3 target gene Myc and Cyclin D1. Together, these observations showed that ROCK2 induces the development of epilepsy, potentially through the activation of astrocyte cell cycle progression via Stat3 pathway.

Methods

Human tissue specimens

All patients were diagnosed with TLE based on having recurrent epilepsy and being refractory to the maximal doses of at least 3 AEDs. All patients with mesial TLE with hippocampal sclerosis were treated surgically in Department of Neurosurgery, Tangdu Hospital, Fourth Military Medical University (Fig 2A.). All hippocampal specimens were randomly selected from an epilepsy brain bank. The criteria, informed consent, brain tissue processing, and how to match control groups were referenced in our previous publications¹⁷⁻¹⁹. The individual patient details are listed in Tables 1 and 2.

Table 1
Clinical characteristics of TLE patients

No	Age(y)/sex	SF(/w)	Course (y)	Seizure type	AEDs before surgery	Surgical approach	Pathology	Follow-Up duration (y)	Surgical Outcome	Application
1	19/M	1-2	14	CPS	VPA, LEV, CZP	LTHAR	FCD,g	5	1	GXP, WB, IHC, MSP
2	16/F	1	15	CPS,SGS	VPA, OXC, LEV,LTG	LTHAR	g	7	1	GXP, WB, IHC, MSP
3	33/M	2-3	17	CPS	LEV,TPM,OXC, CZP	LTHAR	g	6	1	GXP, WB, IHC, MSP
4	25/M	1-2	23	CPS,SGS	VPA, LEV, CBZ, CZP	LTHAR	g,nl	9	1	GXP, WB, IHC, MSP
5	12/M	3-5	8	CPS	VPA,PHT,CBZ,LTG,LEV	LTHAR	FCD,g	10	1	GXP, WB, IHC, MSP
6	15/F	2	11	CPS	VPA,OXC,LTG	LTHAR	FCD,g	6	1	GXP, WB, IHC, MSP
7	9/F	2-3	9	CPS,SGS	OXC, LEV, LTG	LTHAR	g,nl	8	1	GXP, WB, IHC, MSP
8	24/M	4-5	9	CPS,SGS	VPA,OXC,LEV,LTG,CBZ	LTHAR	FCD,g	6	1	GXP, WB, IHC, MSP
9	7/F	7	4	CPS	VPA,OXC,LEV	RTHAR	FCD,g	11	2	WB, IHC, MSP
10	18/M	3-4	7	CPS	VPA,LEV,LTG,OXC	RTHAR	g	5	1	WB, IHC, MSP
11	27/M	1-2	13	CPS	VPA,CBZ,PHT,LEV,LTG	LTHAR	FCD,g,nl	3.5	1	WB, IHC, MSP
12	24/F	2-3	21	CPS,SGS	VPA,LEV,OXC,LTG	LTHAR	g	9	3	WB, IHC, MSP
13	42/F	1-3	27	CPS	VPA,LEV,OXC,LTG	RTHAR	FCD,g	7	1	WB, IHC, MSP
14	15/M	6-7	7	CPS	VPA,LEV,OXC,LTG,PHT	LTHAR	FCD,g	6	2	WB, IHC, MSP
15	23/F	7-14	12	CPS,SGS	LEV, OXC, LTG	RTHAR	g,nl	5	1	WB, IHC, MSP
16	25/F	7	12	CPS	VPA, LEV, LTG, TPM	LTHAR	FCD,g	4.5	2	WB, IHC, MSP
17	17/F	2-4	11	CPS,SGS	VPA, CBZ, LTG	RTHAR	g	6	2	WB, IHC, MSP
18	10/M	3-4	5	CPS	VPA, OXC, LEV, TPM	RTHAR	g,nl	8	1	WB, IHC, MSP
19	11/M	3	11	CPS,SGS	VPA, OXC, LEV	RTHAR	FCD,g	4	2	WB, IHC, MSP
20	33/F	1-2	11	SGS	OXC, LEV, TPM	LTHAR	FCD,g	5	3	WB, IHC, MSP
21	35/F	2-3	30	CPS,SGS	VPA, CBZ, CZP, PHT	LTHAR	g,nl	6.5	2	WB, IHC, MSP
22	20/M	4-6	9	CPS,SGS	VPA, LEV, OXC	RTHAR	FCD,g	7	1	WB, IHC, MSP
23	19/M	7	15	CPS	VPA, CBZ, TPM, LTG	LTHAR	g	3	2	WB, IHC, MSP
24	26/F	7-14	17	CPS	VPA, LEV, OXC, LTG	LTHAR	g,nl	5	2	WB, IHC, MSP

25	27/M	7	22	CPS,SGS	VPA, LEV, LTG, TPM	RTHAR	FCD,g	6	1	WB, IHC, MSP
26	32/F	3-4	13	CPS,SGS	VPA, LTG, LEV, OXC	RTHAR	FCD,g	8	3	WB, IHC, MSP
27	26/M	4	19	CPS	VPA, CBZ, CZP, PHT	RTHAR	g,nl	4.5	2	WB, IHC, MSP
28	23/F	1-3	22	SGS	LTG, OXC, LEV	LTHAR	g	5.5	1	WB, IHC, MSP
29	27/M	7	16	CPS,SGS	VPA, LEV, LTG	RTHAR	g,nl	4	1	WB, IHC, MSP
30	16/F	2-3	10	CPS	VPA,LTG,OXC,LEV,CZP	LTHAR	g	5	2	WB, IHC, MSP
31	28/F	7-14	14	CPS,SGS	LTG, VPA, OXC, LEV	RTHAR	FCD,g	4	1	WB, IHC, MSP
32	26/M	3-4	23	CPS	VPA, OXC, CZP, LEV	LTHAR	FCD,g	6.5	2	WB, IHC, MSP
33	35/M	2-3	31	CPS	VPA, CBZ, CZP, LEV	RTHAR	g	3	3	WB, IHC, MSP
34	31/F	7	10	CPS,SGS	VPA,OXC,LTG,CZP,LEV	RTHAR	FCD,g	4.5	2	WB, IHC, MSP
CBZ, carbamazepine; CPS, complex partial seizure; CZP, clonazepam; FCD, focal cortical dysplasias; GXP, gene expression profiling; IHC, immunohistochemistry; LEV, levetiracetam; LTG, lamotrigine; LTHAR, left anterior temporal lobe hippocampal amygdala resection; RTHAR, right anterior temporal lobe hippocampal amygdala resection; MSP, methylation specific PCR; OXC, oxcarbazepine; PHT, phenytoin; SGS, secondarily generalized seizure; VPA, valproate; TPM, topiramate; WB, western blotting; nl, neuron loss; g, gliosis; F, female; M, male; SF, seizure frequency; w, week; y, year.										

Table 2
Clinical data from 12 control subjects

No.	Gender	Age (y)	Cause of death	Etiology diagnosis	Resection tissue	Histological examination	Seizure	Application
1	M	15	Car accident	N/A	LH	n	None	GXP, WB, IHC, MSP
2	F	26	Car accident	N/A	LH	n	None	GXP, WB, IHC, MSP
3	M	18	Drowning	N/A	LH	n	None	GXP, WB, IHC, MSP
4	M	24	Car accident	N/A	LH	n	None	WB, IHC, MSP
5	M	37	N/A	Severe TBI	RH	n	None	WB, IHC, MSP
6	F	46	N/A	Severe TBI	RH	n	None	WB, IHC, MSP
7	M	35	N/A	Severe TBI	LH	n	None	IHC
8	M	45	N/A	RTG	RH	n	None	IHC
9	M	53	N/A	LTG	LH	n	None	IHC
10	F	44	N/A	LTG	LH	n	None	IHC
11	F	38	N/A	RTG	RH	n	None	IHC
12	F	49	N/A	LTG	LH	n	None	IHC
LTG, left temporal glioma; RTG, right temporal glioma; LH, left hippocampus; RH, right hippocampus; TBI, traumatic brain injury; GXP, gene expression profiling; IHC, immunohistochemistry; MSP, methylation specific PCR; WB, western blotting; l, left; r, right; F, female; M, male; n, normal.								

Animals and Treatments

Adult male SD rats (200–240 g body weight) were obtained from the Experimental Animal Center of the Fourth Military Medical University. Preparation of the lithium–pilocarpine model of temporal lobe epilepsy and how animals were grouped were described in detail in our previously published articles^{17,18}.

Human mRNA profiling

Blocks of hippocampus chosen randomly from the epileptic (n=8) and control (n=3) tissue groups were cut into small pieces and homogenized in 1.5ml Trizol (Sigma). All samples underwent the same tissue dissection and RNA extraction sample preparation protocol performed by the same individuals. Spectrophotometric evaluation of RNA quality and quantity using a microvolumetric Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was verified by gel electrophoresis. The TruSeq® RNA LT Sample Prep Kit v2 (Illumina Inc., USA) was used for high-throughput strand-specific mRNA library preparation, and the qualified libraries were sequenced by Genergy Biotechnology Co. Ltd. (Shanghai, China) using Illumina HiSeq 2500 following the manufacturer's instructions (Illumina)²⁰. All sequencing and data analysis were conducted by Genergy Inc (Shanghai, China). A detailed description of the sequencing process is provided in the supplemental material.

Surgery and intracranial EEG recording

For EEG surgery, adult male SD rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Stainless steel screws were used as electrodes, placed on top of the dura through small holes drilled through the skull and then fixed to the skull using dental cement. The locations of the electrodes were F3/4 (+2.0 mm from bregma, 2.25 mm lateral from midline), C3/4 (-3.0 mm from bregma, 2.75 mm lateral from midline), P3/4 (-7.0 mm from bregma, 2.75 mm lateral from midline) (Fig. 4B). The ground electrode was placed in the midline approximately 1.5 mm posterior of Lambda. EEG electrodes were wired to a head mount that was also fixed to the skull using dental cement (Fig. 4C). After 5–7 days of recovery from the surgery, EEG signals and synchronized video were recorded using the EEG-9200K (Nihon Kohden, Tokyo, Japan). The voltage differential between the pair of electrodes from each brain hemisphere were amplified with a high pass filter (1Hz) and recorded. EEG signals were sampled at 400 Hz and videos were recorded at 30 frames/sec. The detailed process is described in our previously published articles^{21,22}.

Li-Pilo-induced SE and Behavioral grading of seizures

After 5 min of EEG and video recording of baseline activity, rats were first administered a single dose of methylscopolamine (1 mg/kg, i.p.) to allay the peripheral effects of pilocarpine. A single dose of pilocarpine (280 mg/kg, i.p.) was administered approximately 25 min after methylscopolamine to induce acute seizures. After 15 min, rats in the treatment group were injected with Fasudil (25 mg/kg, i.p., APExBio Technology, USA) and returned to their home cages to observe the behavioral grade of seizures. The scoring was based on the Racine scale, as described previously (Racine, 1972) with the following stages: 0, no abnormality; 1, Mouth and facial movements; (2) Head nodding; (3) Forelimb clonus; (4) Rearing; (5) Rearing and falling. A full motor seizure, with temporary loss of postural control, is referred to as a Stage 5 motor seizure.

Cortical Primary Astrocyte Cultures from adult SD Rats

Adult male SD rats from the Experimental Animal Center of the Fourth Military Medical University were sacrificed in a CO2 chamber according to current Fourth Military Medical University (Animal Use for Research and Education Committee) guidelines. Cerebral cortex was dissected aseptically and the meninges removed. Cortices were dissociated into a cell suspension using mechanical digestion. Cells were plated in 6 well plates pre-coated with poly-L-lysine at a concentration of 5×10^5 cells/cm² in modified DMEM/F12 medium [10% FBS, 15 mM HEPES, 14.3 mM NaHCO₃, 1% fungizone and 0.04% gentamicin]. The first change of medium was performed after 24 h of culture. During the first week, the medium change occurred once every 2 days and, from the second week, once every 4 days. From the third week onwards, the cells received medium supplemented with 20% FBS^{23,24}. Around the third to fourth weeks, cells reached confluence and were used for the experiments. The purity of the primary astrocyte cultures was assessed by immunocytochemistry for GFAP.

Plasmid, shRNA and virus

The ROCK2 shRNA sequence used to increase ROCK2 expression was 5'-ACATGCCAGGTGGAGACCTTGTTAATTGCTCTTTTGG-3' (Shanghai GenePharma). Scramble was used as negative control. ROCK2-overexpressing lentivirus were packaged in Hanbio Biotechnology (China).

Double immunofluorescence

Immunofluorescence was performed as described in our previously published articles^{25, 26}. The brain sections were incubated in a primary antibody solution containing rabbit anti-ROCK2 antibody (1:700; #PA5-78290, Invitrogen) and mouse anti- NeuN antibody (1:1000; #ab104224, Abcam) or mouse anti-GFAP antibody (1:1000; #ab10062, Abcam) that was dissolved in the rinse buffer for 12 h at 4 °C. Before secondary antibody incubation, the sections were washed 3 times for 30 min in PBS. The sections were then incubated in a secondary antibody solution containing FITC-conjugated goat anti-rabbit IgG (1:2000; #ab6717, Abcam) and Texas Red-conjugated goat anti-mouse IgG (1:2000; #ab6787, Abcam) in the rinse buffer for 4 h at 4 °C. After washing 3 times for 30 min in PBS, the sections were coverslipped with anti-fading medium. Images of double immunofluorescence staining were obtained using a confocal laser-scanning microscope system (Nikon C2+, Tokyo, Japan). Images were analyzed with ImageJ software (ver. 1.46; NIH, USA). The ratios of cell number of single-positive cells and double-positive cells (%) was evaluated as described in the article²⁷.

Western Blot Analysis

Total proteins from tissues or cell extracts (50 µg/gel lane) were separated by SDS-PAGE, blotted, and probed with anti-ROCK2 (1:800; #PA5-78290, Invitrogen), anti-Stat3 (1:3000; #ab68153, Abcam), anti-p-Stat3 (1:2000; #ab76315, Abcam), anti-β-actin (1:5000; #ab8227, Abcam), and anti-Tubulin (1:5000; #ab6160, Abcam) alone or in combination. Secondary antibodies conjugated with HRP (1:3000, Abcam, UK) were detected using the ChemiDoc XRS+ system (Bio-Rad, Hercules, USA). Bands were quantified by densitometry using ImageJ software (ver. 1.46; NIH, USA).

qPCR

Extract the RNA and generate complementary DNA through GoScript Reverse Transcription System (Promega). The qPCR assay was performed as described previously²⁸. Primers used for qPCR analysis of ROCK2, c-Myc, Cyclin D1, GFAP, P21, P15, and β-actin are listed on Table 3.

Table 3
Oligonucleotide primers for real-time RT-PCR

mRNA target	Sense/anti-sense
ROCK2	TGCCCGATCATCCCCTAGAA AGGCAGTTAGCTTGTTTGT
c-Myc	GAGGTGGAAAACCCGACAGT GAAATAGGGCTGCACCGAGT
Cyclin D1	TAGGGCTGGTAGCATGAGGT AGATCCCGGTGGTACGAGAA
GFAP	TTGACCTGCGACCTTGAGTC GAGTGCCTCCTGGTAACTCG
P21	TGTGATATGTACCAGCCACAGG CGAACAGACGACGGCATACT
P15	GACTCACTCGCGGCTCC GGCTCCCGTTAGACACTCTC
β-actin	CTCTGTGTGGATTGGTGGCT CGCAGCTCAGTAACAGTCCG

Flow Cytometric Analysis of the Cell Cycle

Cell cycle assay was performed as described previously²⁹.

ChIP

The ChIP analysis was performed using the ChIP Assay kit (Upstate Biotechnology, Charlottesville, VA). 10⁷ cells were crosslinked with 1% formaldehyde for 10 min at 37 °C and then washed, lysed, and sonicated to generate 200–500 bp chromatin fragments. The samples were

precleared with 60 µl of salmon sperm DNA–protein A-agarose and subsequently incubated at 4 °C overnight with 2 µg Stat3 antibody and rabbit IgG as control. Immunocomplexes were recovered, washed thoroughly, and eluted with the ChIP elution buffer. Following the reversal of crosslinks at 65 °C for 4 hours, samples were extracted with phenol/chloroform, precipitated with ethanol, and then used as templates for PCR amplification. The sequences of PCR primers detecting c-Myc and Cyclin D1 promoter are available on request.

Methylation Assay

The methylation assay were performed as previously described²⁹. Primers for the methylated promoter region were 5'-GATATACGATAGGAATTACGGGG-3' (sense) and 5'-TCTCTCTACCTTATCTAACCCG-3' (antisense), and for the unmethylated region 5'-GATATATGATAGGAATTATGGGG-3' (sense) and 5'-CTCTACCTTATCTAACCCATTCCC-3' (antisense). The intensity of the methylated and the loading control products were analyzed by ImageJ software (NIH, Bethesda, MD) and compared with the base pairs of the amplicons and the cycles of individual PCR reactions. When the relative ratio of the methylated to the total DNA was more than 50 %, methylation was considered high, whereas it was considered low when the ratio was less than 50 %. Samples with no obvious methylation bands were considered as having no methylation.

IHC

Epileptic and control hippocampus tissues were collected at the tangdu Hospital of Fourth Military Medical University. The immunohistochemistry staining assay was performed and scored as previously described¹⁷. The primary antibodies for anti-ROCK2 (1:200, #PA5-78290, Invitrogen) and anti-p-Stat3 (1:150; #ab76315, Abcam) were applied. The protein levels were statistically analyzed by Student's t-test. Linear regression and Pearson's correlation significance were used to analyze ROCK2 and p-Stat3 correlation.

Statistical analysis

The data are expressed as the mean ±SEM. Statistical analyses were performed in GraphPad Prism 8.0 (GraphPad Software Inc, San Diego, CA). One-way ANOVA analysis followed by Dunnett's multiple comparison test was used to determine the differences among multiple groups. Correlations between the protein levels of ROCK2 and p-Stat3 were assessed using Pearson's rank correlation test. Statistical significance was based on a value of $P < 0.05$.

Results

mRNA Profiling of human refractory epilepsy samples

To gain mechanistic insight into system-level differences between normal and epileptic hippocampus tissues, we determined the gene expression profiles in 8 drug-resistant epilepsy samples and 3 control samples (Tables 1 and 2). Compared with the control samples, 1389 genes were downregulated and 1014 genes were up-regulated in refractory epilepsy samples (Fig. 1A, 1B). Further, we analyzed the top20 upregulated genes in epilepsy samples. Intriguingly, ROCK2 were found most dramatically highly expressed in epilepsy samples (Fig. 1C), suggesting the high levels of ROCK2 might be associated with epilepsy generation.

Elevated expression of ROCK2 in human epileptic hippocampus by the hypomethylation of ROCK2 promoter

To evaluate the role of ROCK2 in epilepsy, we firstly analyzed the expression levels of ROCK2 in the normal (n=6) and epileptic (n=34) hippocampus tissues (Tables 1 and 2). Intriguingly, the densitometric analysis of western blots shows that ROCK2 was highly expressed in the hippocampus tissue of drug-resistant TLE patients (Fig.2B). The ROCK2 product OD in patients was significantly higher than that in controls (Fig. 2C). To determine the underlying mechanisms, we examined the potential changes in ROCK2 gene promoter region. Samples from TLE patients and control tissue samples were collected for methylation analysis by nested PCR. Methylation-specific PCR (MSP) analysis shows that ROCK2 promoter region was highly methylated in control samples and only 1 of 6 TLE patients (Fig.2D). Further, a large scale of MSP analysis identified the hypermethylation of ROCK2 promoter in 9 of 12 normal tissues and 6 of 34 TLE patients (Fig.2E). Thus, epileptogenesis appears to be negatively associated with the hypomethylation of ROCK2 promoter and concomitant induced its expression.

Increased expression of ROCK2 in hippocampal and neocortical epilepsy

Rats were divided randomly into eight subgroups of ten rats each and injected with lithium and 24 h later with saline (one subgroup) or the convulsant pilocarpine (seven subgroups) to establish a control and spontaneous epilepsy model groups. Neural ROCK2 expression was then determined at different times after seizure induction (1, 3, 5, 7, 15, 30, or 60 days). The lithium–pilocarpine model of epilepsy in rats were prepared and evaluated as previously described¹⁷. Western blot analysis of brain lysates prepared at these different times after pilocarpine treatment revealed increased ROCK2 expression compared with controls (Fig. 3). Compared with the control group, expression of ROCK2 in the rat temporal lobe increased gradually during the acute period (day 1–3) (Fig. 3A) before increasing obviously during the latent period (days 5–15) and the chronic phase (days 30–60). Expression of ROCK2 as expressed by product OD relative to that of β -actin was significantly different between control and every epilepsy subgroup ($p < 0.05$ by Tukey's HSD test) except for the 1-day subgroup ($p > 0.05$) (Fig. 3B). The expression of ROCK2 in epileptic hippocampus (Fig. 3C, D) followed a similar trend to that in the temporal cortex, with significant pair-wise differences in ROCK2 expression between the control and every epilepsy subgroup ($p < 0.05$) except the 1-day subgroup. The expression of ROCK2 in the hippocampus increased even more obviously at 15, 30 and 60 days after seizures ($p < 0.01$) (Fig. 3D).

Fasudil alleviates rat epileptic seizures *in vivo* and protects against Li-Pilo-induced SE

Li-Pilo-induced epileptic rat model is one of the most widely used on epileptogenesis research and has been assess the seizures based on changes in the Racine stages. The rats were then divided into 3 groups (saline group, PILO group and Fasudil treatment group). Fasudil, a specific Rho-kinase inhibitor, was used to treat the rats following Li-Pilo induced seizures for 2 weeks. Then seizures were induced by the low dose PILO (200 mg/kg, i.p.) again and assessed the effects of Fasudil on the epileptogenesis. In the present study, the video-EEG recording and racine stages were used to analyze seizure severity between Fasudil treatment group and PILO group. After administration of PILO, stage 3 behavioral scores earlier appeared in PILO group compared with Fasudil treatment group, stage 4 and 5 behavioral scores did not appear in Fasudil treated rats (Fig. 4A). Further, we examined hippocampal EEG activity in LiCl-Pilo epileptic rats at symptomatic stages (Fig. 4D). The latency (min) of generalized tonic-clonic seizures (GTCS) in the PILO group was 28.39 ± 2.10 , while the latency in the Fasudil treatment group was 53.01 ± 4.16 (Fig. 4E). The EEG showed 14.96 ± 0.43 spikes per second in the PILO group and 6.88 ± 0.13 spikes per second in the Fasudil treatment group (Fig. 4F). The spike amplitude (μ V) of EEG in PILO group and Fasudil treatment group were 232.83 ± 13.61 and 108.72 ± 2.94 , respectively (Fig. 4G).

ROCK2 activates Stat3 pathway in Pilocarpine-Treated Epileptic Rats

To further investigate the role of ROCK2 in regulation of epileptogenesis, we determined the downstream targets in SE rat model. JAK/Stat3 pathway was previously shown constitutively activated during brain injuries like SE, and plays a key role in epilepsy development^{30,31}. In the present study, we observed the phosphorylation and activation of Stat3 following SE. Intriguingly, Fasudil administration significantly decreased Stat3 phosphorylation, indicating ROCK2 potentially contributes to epileptogenesis through the regulation of Stat3 pathway (Fig. 5A, 4B). Further, we determined the downstream target gene expressions of Stat3. Myc and Cyclin D1, which play important roles in regulating cell proliferation and cell cycle progression, were induced following SE and significantly decreased after Fasudil treatment (Fig. 5C). Therefore, we suppose that ROCK2 induces cell proliferation *via* the activation of Stat3 pathway in brain tissue, and further contributes to the development and progression of epilepsy.

ROCK2 is predominantly expressed in astrocytes during epileptogenesis

Above data showed the elevated expression of ROCK2 in the brain tissues of TLE patients. To further address ROCK2 function in epileptogenesis, we detected the localization of ROCK2 in both neuron and astrocytes of rat brain tissue. The co-localization of ROCK2 with NeuN or GFAP was observed in normal brain tissue, indicating ROCK2 expression in certain level is necessary for the biological function of both neuron and astrocytes (Fig. 6A, 6C). Whereas, from the third day after SE, ROCK2 expression in astrocytes was significantly increased in contrast with the constant level of ROCK2 in neuron tissue. Quantitative analysis showed nearly 2-fold increase of ROCK2 expression in astrocytes (Fig. 6B, 6D). Thus, we show that ROCK2 is predominantly expressed in astrocytes during epileptogenesis, indicating ROCK2 possibly induces epilepsy progression through the specific regulation of astrocyte function.

ROCK2 induces epileptogenesis by activating astrocyte cell cycle progression via Stat3 pathway

Since accumulated evidence showed that astrogliosis plays important roles in epileptogenesis, it is reasonable to consider the involvement of ROCK2 in astrocyte proliferation and activation^{32,33}. Based on this hypothesis, we set up the primary astrocytes from rat brain tissue, whose phenotype has been confirmed with GFAP in our previous study¹⁷. To investigate how ROCK2 confers astrocyte behavior, we overexpressed ROCK2 in astrocytes. Expectedly, the phosphorylation of Stat3 was dramatically induced by ROCK2 expression (Fig. 7A). Further, GFAP, the marker

of astrocyte proliferation potential and Stat3 target gene, was induced by ROCK2 (Fig. 7B). During cell cycle analysis, we found ROCK2 accelerated astrocyte G1-S transition, indicating ROCK2 induces astrocyte proliferation which confers gliosis potentially through the regulation of cell cycle progression (Fig. 7C). Accordingly, Fasudil treatment blocked ROCK2 induction of pStat3 and GFAP expressions, and G1-S transition, which further confirmed our hypothesis (Fig. 7A-C).

To fully address the related mechanism, we then determined whether ROCK2 regulates cell cycle transition via Stat3 pathway. Previous data revealed Stat3 target genes, Myc and Cyclin D1, were ROCK2 dependently induced in SE rat brain tissue (Fig. 5C). Further study showed that Myc and Cyclin D1 were remarkably induced by ROCK2 in astrocytes (Fig. 7D). The CDK inhibitor p21 and p15, which are Myc direct targets, were decreased after ROCK2 overexpression. Additionally, ChIP experiments indicated that the binding of Stat3 to Myc and Cyclin D1 promoter was dramatically increased by ROCK2 overexpression (Fig. 7E). In the presence of Fasudil, ROCK2-induced expressions and Stat3 occupancies of Myc and cyclin D1 were significantly abolished in astrocytes (Fig. 7D, 7E). Taken together, these data suggest that ROCK2 induces the development of epilepsy, potentially through the activation of astrocyte cell cycle progression via Stat3 pathway.

ROCK2 is associated with Stat3 activation in TLE patients

To evaluate the clinical significance of ROCK2 induction of Stat3 pathway during epilepsy, we performed immunohistochemical analysis to assess the expressions and association of ROCK2 and pStat3 in TLE patients. The results revealed that both ROCK2 and pStat3 expressions were increased in patient samples (n=34) compared with control samples (n=12) (Fig. 8A). Indeed, in patient tissues versus control, ROCK2 levels were 7.82 versus 3.92 (Fig. 8B), respectively, and pStat3 levels were 6.88 versus 2.67 (Fig. 8C), respectively. An association study showed that ROCK2 expression positively correlated with pStat3 expression in the entire sample population (Fig. 8D) ($p < 0.001$, ANOVA). Thus, the epilepsy-associated increase of ROCK2 is associated with the increased surface activation of Stat3 pathway.

Discussion

Active RhoA binds to its downstream targets to initiate numerous cellular responses. ROCK, a serine/threonine kinase, is one of the most studied RhoA effectors and is involved in regulation of the actin cytoskeleton, cell proliferation, focal adhesion /stress fiber formation, and smooth muscle contraction³⁴. The current study explored the role of ROCK2 in epilepsy, focusing on the possibility that ROCK2 inhibition is required for the suppression of seizures. Although ROCK2 has not previously been suspected to play a role in epilepsy, our data demonstrate the importance of ROCK2 in the Li-Pilo model and clinical epilepsy sample, by the inhibition of ROCK2 associated reduction in seizure activity. Thus, we identify ROCK2 as a key regulator of epilepsy, and a potentially novel therapeutic target for the prevention and treatment of epilepsy.

In this study, ROCK2 expression was firstly evaluated in normal and epileptic brain tissue. We show that increased ROCK2 expression in human epileptic brain tissue. The underlying mechanism study suggests that ROCK2 gene promoter region was hypomethylated in TLE patients, which potentially contributes to the elevated expression of ROCK2. To our knowledge this is the first study providing direct evidence of increased ROCK2 expression in patients with drug-resistant TLE. Further, the highly isomorphic with human cases of TLE animal model was used, namely the model of TLE with pilocarpine-induced seizures³⁵. In order to analyze the dynamics of epileptic seizure-induced alterations on the brain, samples taken from seizure experiencing animals 1, 3, 5, 7, 15, 30, and 60 days after proconvulsive agent administration were examined. Our study shows that the expression of ROCK2 protein increased dramatically in epilepsy rat cortex and hippocampus. Thus, we set up the association of ROCK2 with epileptogenesis.

Astrocytes are now known to be intimately involved in the pathogenesis of many neurological disorders, including epilepsy^{32,36}. The reactive astrocytosis may facilitate the development of epileptic seizures³⁷. The cells of the high ROCK2 expression were found in pyramidal neurons of the hippocampus and cerebral cortex and Purkinje cells of the cerebellum¹⁰. By immuno-fluorescence, ROCK2 was mainly localized in the neurons in our study. A previous study has shown that RhoA activation in the cortex and hippocampus 24 h after traumatic brain injury-related seizures⁴. In this study, we observed the expression of ROCK2 in hippocampal astrocytes in the Li-Pilo model. GFAP is the main intermediate filament protein in mature astrocytes. It has been demonstrated that GFAP-immunoreactive reactive astrocytes exhibited markedly increased RhoA expression in response to spinal cord injuries after kainic acid treatment³⁸. We show reactive astrocyte co-localization with ROCK2 expression in the Li-Pilo-treated hippocampus, which is consistent with the Rho-ROCK pathway induces the generation of a reactive astrogliosis³⁹. However, the expression of ROCK2-positive neurons is not induced by Li-Pilo treatment, indicating the increased expression of ROCK2 may be associated with pathological changes predominantly in astrocytes.

JAK/Stat3 pathway plays an important role in normal development and a number of disease processes, including epileptogenesis^{30,40,41}. Recent evidence shows the aberrant activation of this pathway after a variety of brain insults that can lead to epilepsy, such as pilocarpine or kainate-induced SE^{30,40}, and traumatic brain injury⁴². Whereas, the potential mechanism has not been fully addressed. Our study shows the highly expression ROCK2 can activate Stat3 activation in astrocytes, and further contribute to the development of epilepsy via the induction of astrogliosis. Thus, inhibiting ROCK2 expression or Stat3 phosphorylation may be a therapeutic approach to inhibit primary epileptogenesis.

Because ROCK is a serine/threonine kinase and cannot directly mediate tyrosine kinase phosphorylation of Stat3, it is likely that other intracellular signaling components, which may interface between ROCK and STAT3, are also engaged in this ROCK2-dependent activation of Stat3^{43,44}.

The transcription factor Stat3 is known to have important roles in regulating gene expression, specifically increasing genes important to cell proliferation and cell cycle progression^{31,45}. In this study, we determined how ROCK2 induces epilepsy via Stat3 pathway, and found Stat3 target genes, Myc and Cyclin D1, were significantly induced by ROCK2. Further, ROCK2 increased the occupancies of Stat3 on Myc and Cyclin D1 promoters, and accelerated astrocyte cell cycle progression. The CDK inhibitor protein p21 and p15 are important downstream targets of Myc and critical for cell cycle inhibition^{46,47}. We show here that p21 and p15 were further suppressed by ROCK2 activation of Myc via Stat3 pathway. Thus, we show ROCK2 induces astrocyte cell cycle progression in regulation of Stat3 pathway, and further contribute to the development of epileptic seizures. These experiments may explain, on one hand, the requirement of Stat3 for ROCK2-mediated activation of astrogliosis and, on the other, the ability of ROCK2 to provide a second input of cell cycle regulation for epileptogenesis in addition to the regulation of actin cytoskeleton dynamics and glutamatergic synaptic function as mentioned before^{48,49}.

Since the continuous activation of astrocytosis confers epileptogenesis, our study provides the evidence of ROCK2 involvement in astrogliosis, which suggests ROCK2 could be considered as a potential target for the treatment of TLE patients. Further, we aimed to elucidate the role of ROCK2 activation of Stat3 pathway in the development and progression of epilepsy, specifically whether brief inhibition of ROCK2 affects severity of SE. We thus introduced ROCK2 inhibitor Fasudil in the present study. Fasudil has been a commonly used rock signal inhibitor, which significantly reduced the duration of tonic hindlimb extensions and recovery latency for righting reflex in maximal electroconvulsive shock induced epilepsy mice, and prolonged the onset time of seizures in PTZ epilepsy mice¹⁵. Song et al. found that FH is a potential treatment option to prevent or reverse cognitive dysfunction in Li-Pilo-induced epileptic rat model⁵⁰. Furthermore, Çarçak et al. have demonstrated a single systemic administration of Fasudil partially reduced the duration and frequency of absence seizure⁵¹. In the present study, we confirmed that the Racine scale score of the Fasudil treatment group was significantly lower than that of the PiLo group. Fasudil prolonged the latency of generalized seizures and reduced the amplitude and frequency of EEG. Based on the results of this article and the clinical practice of our epilepsy center (data to be published), we believe that ROCK2 inhibitors may have better therapeutic effects on GTCS and SE.

Furthermore, immunohistochemical staining of epileptic samples for ROCK2 and pStat3 also showed a correlation of protein accumulation between these two proteins, which is also positively correlated with the development of epilepsy. Thus ROCK2-mediated activation of Stat3 pathway likely serves as an important guardian for epileptogenesis. It should be noted that, as is often observed among clinical samples, no 1:1 correlation between ROCK2 and pStat3 expression in tissues was observed. Thus, in addition to ROCK2, other factors may also contribute to the activation of Stat3 pathway during epilepsy progression.

Taken together, our work provides the evidence that elevated expression of ROCK2 due to its promoter hypomethylation may contribute to the etiology of epilepsy. In terms of epileptogenesis, ROCK2 accelerates astrocytes cell cycle progression via the activation of Stat3 pathway, and further induces astrogliosis. As such, Targeting ROCK2 may lead to the inhibition of Stat3 phosphorylation in astrocytes, and that will be especially effective at restricting epilepsy progression. These data will extend the role of ROCK2 in the pathophysiology of drug-resistant TLE and define ROCK2 and its downstream signaling pathways as potential targets for therapeutic intervention for drug-resistant TLE.

Abbreviations

TLE, Temporal lobe epilepsy; Li-Pilo, Lithium-pilocarpine; CNS, Central nervous system; ROCK, Rho-associated coiled-coil forming protein kinase; PTZ, Pentylentetrazole; MES, Maximal electroconvulsive shock; IHC, Immunohistochemistry; CDK, cyclin-dependent kinase; AEDs, Anti-epileptic drugs; SD, Sprague Dawley; EEG, Electroencephalography; SE, Status epilepticus; FBS, Fetal bovine serum; NeuN, Neuronal nuclei; GFAP, Glial fibrillary acidic protein; HRP, horseradish peroxidase; qPCR, Quantitative PCR; ChIP, Chromatin immunoprecipitation; FLAIR-MRI, Fluid-attenuated inversion recovery magnetic resonance imaging; FDG-PET; Fluorodeoxyglucose-positron emission tomography; GTCS, Generalized tonic-clonic seizures.

Declarations

Authors' contributions

BL and LLS designed the research and wrote the manuscript; BL and LLS performed the study supervision. LJS, HZ and JGJ conducted the experiments, analyzed the data and wrote the manuscript; CW, XPQ, XJ, LG, GL and DLW participated in scientific discussions, analyzed the data and provided detailed feedback on all areas of the project. All authors read and approved the final manuscript.

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Availability of data and materials

The data used in our study are available from the authors on reasonable request.

Ethics approval and consent to participate

All the animal experiments were approved by the Local Committees of the Use of the Laboratory Animals, Fourth Military Medical University (Xi'an, China) and were performed in accordance with the Guidelines for Animal Experimentation of the Fourth Military Medical University and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The human experimental protocols were approved by the Committee on Human Research at the Fourth Military Medical University. All procedures were in accordance with the principles of the Helsinki Declaration. All patients received a full description of the study and gave written informed consent.

Consent for publication

Not applicable.

Competing Interests

The authors declare no potential conflict of interest.

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Figures

Figure 1

mRNA Profiling of human refractory epilepsy samples. (A-B) Heatmap (A) and volcano plot (B) analysis were performed to determine the genes differentially expressed in 8 drug-resistant epilepsy samples and 3 control samples. (C) The top20 upregulated genes were identified and analyzed by heatmap.

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Figure 2

Elevated hippocampal ROCK2 expression by the hypomethylation of ROCK2 promoter. (A) Patient 3's pre-operative assessment and surgical resection specimens: coronal FLAIR-MRI of the head showed abnormal high signal intensity in the left hippocampus (left), FDG-PET revealed hypometabolic lesions in the left mesial temporal lobe (middle), interictal electroencephalography showed positive sharp slow waves in the left mesial temporal region (below), and the anterior temporal lobe, hippocampus and amygdala were immediately stored in the epilepsy brain bank after surgical resection (right). (B) Representative western blot assays of ROCK2 expression in the hippocampus of controls and patients with TLE. (C) Comparison of the Western blot intensity ratios of ROCK2/ β -actin in the hippocampus of controls (n=6) and patients with TLE (n=34). (D) Representative methylation status of the ROCK2 promoter region in the hippocampus of controls and patients with TLE. (E) Statistical graphs show the comparison of the hypermethylation of ROCK2 promoter in the hippocampus of controls (n=12) and patients with TLE (n=34). The experiments were repeated 3 times. P patients with epilepsy, C control. Data were analyzed using unpaired t-tests. **P < 0.01.

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Figure 3

Increased ROCK2 Expression in the temporal cortex and hippocampus of LiCl-PILO epilepsy Rats. (A, C) Representative western blot assays of ROCK2 expression in the temporal cortex and hippocampus at different post-seizure time points. (B, D) Compared with the control group, the expression of ROCK2 in temporal cortex and hippocampus of PILO group increased significantly from the third day after status epilepticus. The experiments were repeated 3 times with 3 rats in each group. Data were analyzed using one-way ANOVA and Dunnett's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$.

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Figure 4

Fasudil reduces epileptic seizures in LiCl-PILO epilepsy Rats. (A) PILO-induced status epilepticus was scored every 5 minutes for up to 4 h according to a modified Racine scale. Average scores in the Fasudil treatment group were lower than the PILO group. (B, C) The location of intracranial electrodes and its monitoring environment in LiCl-PILO epileptic rats. (D) The representative hippocampal EEG traces at 1 h after pilocarpine administration were showed. (E) The latency of GTCS in the Fasudil treatment group tends to be longer than in the PILO group. (F, G) Compared with PILO group, the spike amplitude and frequency of hippocampal EEG were significantly attenuated in Fasudil treatment group.

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Figure 5

ROCK2 activates Stat3 pathway in Pilocarpine-Treated Epileptic Rats. (A-B) p-Stat3, Stat3 and β -actin protein levels were determined (A) and quantified in the brain tissues of rats in indicated group. (C) c-Myc and Cyclin D1 mRNA levels were determined in the brain tissues of rats in indicated group. Data are expressed as means $\pm$ SD (n = 3).

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Figure 6

Cellular localization of ROCK2 in the LiCl-Pilo epilepsy rats' CA1 regions. Double immunofluorescence labelling for ROCK2 in the epileptic rats' CA1 regions during acute post-seizure phases. (A) Expression of ROCK2 and the neuronal marker NeuN in the CA1 regions. (C) Expression of ROCK2 and the glial marker GFAP in in the CA1 regions. (B, D) From the 3th day after the status epilepticus, the number of ROCK2-positive astrocytes was significantly higher than that of the control group, while the expression of ROCK2-positive neurons was not changed. *P<0.05 by Student's t test compared to control (n = 6 per group). Scale bars = 62.5 μm.

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Figure 7
ROCK2 induces epileptogenesis by activating astrocyte cell cycle progression via Stat3 pathway. (A-B) Astrocytes were infected with ROCK2 over-expression lentivirus, and then treated with 5 μ M Fasudil for 24 h. (A) The indicated protein levels (A), GFAP mRNA levels (B), cell cycle progression (C) were determined in indicated groups, respectively. (D-E) Stat3 target gene mRNA levels (D) and Stat3 enrichments on Myc or Cyclin D1 promoter were determined respectively. Data are expressed as means $\pm$ SD (n = 3).

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Figure 8

ROCK2 is associated with Stat3 activation in TLE patients. (A) Immunohistochemistry staining of ROCK2 and p-Stat3 in normal human and TLE patient tissues. (B, C) Relative expression level of ROCK2 or p-Stat3 in human tissue samples. Student's t-test was applied for statistical analyses. **, P <0.01 (D) Positive correlation between ROCK2 and p-Stat3 expressions with linear regression and Pearson's correlation significance (P <0.0001, ANOVA test).

Figure 8

ROCK2 is associated with Stat3 activation in TLE patients. (A) Immunohistochemistry staining of ROCK2 and p-Stat3 in normal human and TLE patient tissues. (B, C) Relative expression level of ROCK2 or p-Stat3 in human tissue samples. Student's t-test was applied for statistical analyses. **, $P < 0.01$ (D) Positive correlation between ROCK2 and p-Stat3 expressions with linear regression and Pearson's correlation significance ($P < 0.0001$, ANOVA test).