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Upregulations of CRH and CRHR1 in the Epileptogenic Tissues of Patients with Intractable Infantile Spasms

Xiao-Lin Yang, Bing Chen, Xiao-Qing Zhang, Xin Chen, Mei-Hua Yang, Wei Zhang, Huan-Ran Chen, Zhen-Le Zang, Wei Li, Hui Yang & Shi-Yong Liu

Department of Neurosurgery, Second affiliated Hospital, Third Military Medical University, Chongqing, China

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Correspondence
Shi-Yong Liu and Hui Yang, Department of Neurosurgery, Second affiliated Hospital, Third Military Medical University, 183 Xinqiao Main Street, Shapingba District, Chongqing 400037, China.
Tel: +86-23-6875-5610;
Fax: +86-23-6521-8204;
E-mails: liushi24252016@163.com and huiyang_64@sohu.com
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The first two authors contributed equally to this work.

SUMMARY

Aim: Infantile spasms (IS) are an age-specific epileptic syndrome with specific clinical symptom and electroencephalogram (EEG) features, lacking treatment options, and a poor prognosis. Excessive endogenous corticotropin-releasing hormone (CRH) in infant brain might result in IS. However, the data from human IS are limited. In our study, we investigated the expressions of CRH and its receptor type 1 (CRHR1) in surgical tissues from patients with IS and autopsy controls. **Methods:** Specimens surgically removed from 17 patients with IS, and six autopsy controls were included in the study. Real-time PCR, Western blotting, and immunostaining were used to detect the expressions of mRNA, protein expression, and distribution. The correlation between variates was analyzed by Spearman rank correlation. **Results:** The expressions of CRH and CRHR1 were significantly upregulated in the epileptogenic tissues of IS patients compared with the control group. CRH was distributed mainly in neurons, while CRHR1 was distributed in neurons, astrocytes, and microglia. The expression levels of CRH and CRHR1 were positively correlated with the frequency of epileptic spasms. Moreover, the expression of protein kinase C (PKC), which was an important downstream factor of CRHR1, was significantly upregulated in the epileptogenic tissues of patients with IS and was positively correlated with the CRHR1 expression levels and the frequency of epileptic spasms. **Conclusion:** These results suggest that the CRH signal transduction pathway might participate in the epileptogenesis of IS, supporting the hypothesis that CRH is related to the pathogenesis of IS.

Introduction

Infantile spasms (IS) are an age-specific epileptic syndrome characterized by frequent attacks of epileptic spasms, hypsarrhythmia on EEG, and mental retardation, with an unclear pathogenesis. In 1958, Sorel and Dusaucy-Bayloye found that ACTH could treat IS [1] and ACTH has been the first choice of treatment until now. Previous studies have indicated that the levels of ACTH and cortisol were significantly reduced in the cerebrospinal fluid of patients with IS [2] and ACTH maybe play an antiepileptic role by inhibiting the expression of CRH in the amygdala [3,4]. Animal studies have shown that CRH can induce severe seizures and increase the excitability of immature brain [5–7]. Moreover, excessive CRH was considered to induce IS [8]. To our knowledge, reports directly investigating the relationship between CRH and IS in the epileptogenic tissues of patients with IS are lacking.

CRH has two major receptor subtypes in the brain, CRHR1 and CRHR2. The affinity of CRH to CRHR1 is greater than that to CRHR2 [9,10], and CRHR1 is considered more important for CRH for epilepsy. Previous studies indicated that convulsant effect of CRH in the immature brain may be mediated by CRHR1[11], the expressions of CRH and CRHR1 were simultaneously upregulated in the autopsy brain tissues from generalized epilepsy [12], and CRH has been confirmed to induce CRHR1 expression in the cell membrane in the immature brain [13]. CRH receptors are the members of G protein-coupled receptor family, which triggers the activation of cAMP and phospholipase C (PLC) by binding CRH and further activating the intracellular protein kinases A (PKA), protein kinase C (PKC), and other signaling pathways factors [14]. PKC is closely associated with epilepsy. An electrophysiological study indicated that the activated PKC in rat hippocampal neurons plays an important role in the susceptibility to epilepsy and participates in the generation and maintenance of epilepsy [15]. However, the roles of CRH, CRHR1, and the downstream signals in the epileptogenic tissues of human patients with IS remain unclear.

Our study examined the expressions of CRH and CRHR1 in the epileptogenic tissues of patients with IS and analyzed the changes in the downstream signal molecular PKC, to elucidate their roles in the epileptogenesis of IS.

Patients and methods

Patients

The samples in this research were collected from the Neurosurgery Department at Xinqiao Hospital (Third Military Medical University, Chongqing, China). All processes and experiments were implemented under the guidance of the Ethics Committee of the Third Military Medical University. Human cerebral tissues were used according to the Declaration of Helsinki. No brain tissues were removed strictly for research purposes. All patients were independently evaluated by two neurologists. The inclusion criteria included the following: (1) the cases corresponded to the diagnostic criteria for IS [16]; (2) multiple antiepileptic drugs, including ACTH and prednisone (Vigabatrin is not available in mainland China), had poor effects, that is the surgical intervention indications [17–19]; (3) the epileptogenic zone of IS could be precisely localized after sufficient presurgical evaluation [16]. All the results of clinical semiology, EEG, brain MRI, and PET examinations were reviewed separately by independent neurosurgeons. The patients' clinical details are listed in Table 1. Six controls were obtained from autopsies (M/F, 4/2; mean age 2.8 years; range 1–5 years) without a history of epilepsy, stress, or other neurological disorders; all cases was not affected by agonal status before death, death occurred relatively quickly after trauma or disease [20–22]; the postmortem interval was <24 h. The autopsy was reviewed by two neuropathologists; both gross and microscopic observation showed no structural abnormalities. The detailed clinical characters are presented in Table 2.

Tissue Preparation

The brain specimens collected were immediately sectioned into two parts at the time of operation or autopsy. One part was mixed in 10% diluted formalin for 24 h and inserted into paraffin. The paraffin-inserted sample was cut into 5-μm section for hematoxylin/eosin (H&E) staining and immunohistochemistry. The others were put in liquid nitrogen and saved at −80°C for later use in real-time PCR (polymerase chain reaction) and Western blot analyses.

Real-time PCR Analysis

RNA samples prepared from histologically normal brain tissues and epileptogenic tissues of patients with IS were used to perform real-time PCR analysis. Total RNA was isolated from each sample using 1 mL RNAiso plus reagent following the manufacturer's instructions (TaKaRa, Otsu, Japan). The concentration and quality of RNA were assessed by spectrophotometer at 260/280 nm (Ocean Optics, Dunedin, USA). Total cDNA was synthesized using 1 μg of total RNA and an oligo (dT) primer (TaKaRa, Otsu, Japan), and PCR primers were synthesized by TakaRa Company (Dalian, China). The following primers were used: CRH (forward: tccgaggagcctcccatc, reverse: aatctccatgagtttcctgttgc), CRHR1 (forward: gcctctgactcaccacgatg, reverse: tctgatgatgacacctgacttctg), CRHR2 (forward: tcagccgtgaggaagaggtg, reverse: ggccgtctgcttgatgctgt), and β-actin (forward: gcaccacaccttctacaatgagc, reverse: tagcacagcctggatagcaacg). The PCR conditions were as follows: predenaturation at 95°C for 10 s, 40 cycles of denaturation at 95°C for 5 s, and annealing and elongation at 53.9°C for 30 s. Relative quantification of the mRNA expressions of CRH, CRHR1, and CRHR2 was calculated according to the $2^{-\triangle\triangle CT}$ method.

Western Blotting

The frozen surgical specimens of patients with IS and the autopsy specimens of the control cortex were analyzed using Western blotting. All of the samples were homogenized with RIPA buffer including 10% protease inhibitor (Beyotime, Shanghai, China). The protein concentrations were measured using the Protein Quantitation Kit (Abcam, Cambridge, UK). For electrophoresis, 50 μg/lane of protein lysates was isolated by 10% SDS–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membrane (Millipore, Temecula, CA, USA) in glycine transfer buffer. The polyvinylidene fluoride membranes were blocked in 5% non-fat-dried milk for 2 h and incubated overnight with anti-human CRH (rabbit polyclonal, 1:2000; Abcam), anti-human CRHR1 (goat polyclonal, 1:500; Abcam), anti-human PKC (mouse monoclonal, 1:600; Boster, Wuhan, China), anti-human PKA (rabbit polyclonal, 1:400; Boster), and anti-GAPDH (rabbit monoclonal, 1:1000; CST, Beverly, MA, USA) primary antibodies. The next step was washing and incubating with a horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat second antibodies (1:1000; Beyotime) for 1 h at 37°C, the immunoreactivity (IR) was directly viewed by chemiluminescence.

Immunostaining

After some tissue sections were stained with H&E, the remaining sections were used for immunostaining. The sections were deparaffinized in xylene and ethanol solution, rehydrated in deionized water, and then incubated for 30 min in 0.4% H_2O_2 diluted in methanol. All tissue sections were placed into a phosphate-buffered saline solution (0.01 M, pH7.4) and heated in a microwave oven for 20 min over medium high heat to recover the antibody. The sections were incubated with 0.3% Triton-× 100 for 1 h at 37°C, and then were incubated with anti-human CRH (rabbit polyclonal, 1:400; Abcam), anti-human CRHR1 (goat polyclonal, 1:100; Abcam), and anti-human PKC (mouse monoclonal, 1:100; Boster, China) first antibodies overnight at 4°C. Following three washes, the tissue slices were incubated with a secondary antibody combined with a peroxide-labeled dextran polymer (Beyotime) for 1 h at 37°C. The immunoreactions were visualized using prepared 3.5-diaminobenzidine solution (DAB, Beyotime), and then, the tissue slices were counterstained with hematoxylin. After decoloration and dehydration, the sections were viewed under a microscope. We did not observe immunoreactive cells in negative control experiments, including pretermission of the first antibody.

Table 1 Clinical and neuropathological characteristics of patients with IS

Case NO.	Gender	Pathology	Age at seizure onset (month)	Age at surgery (month)	Epilepsy duration (month)	Seizure frequency (per month	Seizure Type	Cortical Resection/ specimens	PO	Brain MRI	Brain PET	EEG (Present)	AEDs	Application in the current study
1	M	FCDIa	6	46	40	300	IS	Lt P-O/P	I	CD	Focal hypometabolism	Variant Hypsarrhythmia, origin of left parietal occipital (ictal phase)	ACTH LEV TPM CLZ VPA PDN	PCR WB IHC
2	M	Atypical gliosis	6	18	12	130	IS	Lt hemisphere/O	II	Multifocal atrophy of unilateral hemisphere	Hypometabolism of unilateral hemisphere	Hypsarrhythmia, multifocal origin of left hemisphere (ictal phase)	ACTH VPA LEV	WB IHC
3	M	FCDIa	7	21	14	120	IS PS	Rt P/P	I	CD	Focal hypometabolism	Hypsarrhythmia, origin of right parietal (ictal phase)	PDN LTG TPM VPA	WB
4	M	Atypical gliosis	4	36	32	210	IS	Lt T-P/T	I	Focal atrophy	Focal hypometabolism	Variant Hypsarrhythmia, origin of left temporal parietal (ictal phase)	ACTH TPM VPA LTG CLZ	PCR WB IHC
5	M	FCDIIa	5	40	35	210	IS PS Tonic	Rt hemisphere/FR	I	CD	Hypometabolism of unilateral hemisphere	Variant Hypsarrhythmia, multifocal origin of right hemisphere (ictal phase)	PDN VPA LEV CLZ LTG TPM	WB IHC
6	M	Normal	5	14	9	240	IS PS	Rt P/P	I	Normal	Focal hypometabolism	Hypsarrhythmia, origin of right parietal (ictal phase)	ACTH CLZ VPA	IHC
7	F	Atypical gliosis	1	10	9	120	IS	Lt P-O/O	II	Focal encephalmalacia	Focal hypometabolism	Hypsarrhythmia, origin of left parietal occipital (ictal phase)	ACTH VPA LEV	WB IHC
8	M	FCDIa	8	34	26	600	IS	Lt T-P-O/O	I	CD	Hypometabolism of unilateral hemisphere	Variant Hypsarrhythmia, origin of left parietal occipital (ictal phase)	PDN LEV VPA CLZ TPM	PCR WB IHC
9	F	FCDIa	30	49	19	900	IS	Rt O/O	II	CD	Focal hypometabolism	Hypsarrhythmia, origin of right occipital (ictal phase)	ACTH LEV TPM	WB IHC

(continued)

Table 1 (Continued)

Case NO.	Gender	Pathology	Age at seizure onset (month)	Age at surgery (month)	Epilepsy duration (month)	Seizure frequency (per month	Seizure Type	Cortical Resection/ specimens	PO	Brain MRI	Brain PET	EEG (Present)	AEDs	Application in the current study
10	M	Normal	12	58	46	300	IS	Lt FR/FR	I	Normal	Focal hypometabolism	Variant Hypsarrhythmia, origin of left frontal (ictal phase)	VPA LMT ACTH CLZ PDN	WB IHC
11	F	FCDIa	3	25	22	600	IS PS	Rt FR-P-O/O	I	CD	Hypometabolism of unilateral hemisphere	Variant Hypsarrhythmia, origin of right parietal occipital (ictal phase)	VPA CLZ LEV PDN	IHC
12	M	Atypical gliosis	1	43	42	300	IS PS Tonic	Lt FR/FR	I	Focal encephalmalacia	Focal hypometabolism	Hypsarrhythmia, origin of left frontal (ictal phase)	PB VPA LEV ACTH CLZ	IHC
13	M	FCDIa	6	39	33	180	IS GTCS	Rt FR/FR	I	CD	Focal hypometabolism	Variant Hypsarrhythmia, origin of right frontal (ictal phase)	VPA VB6 LMT LEV ACTH	PCR WB IHC
14	F	Atypical gliosis	6	23	17	100	IS GTCS Tonic PS	Lt hemisphere/T	III	Multifocal atrophy of unilateral hemisphere	Hypometabolism of unilateral hemisphere	Variant Hypsarrhythmia, multifocal origin of left hemisphere (ictal phase)	VPA CLZ LEV TPM VB6 ACTH	WB IHC
15	M	FCDIa	1	9	8	270	IS PS	Rt T-O/TO	I	CD	Focal hypometabolism	Hypsarrhythmia, origin of right temporal occipital (ictal phase)	VPA PDN ACTH	WB IHC
16	M	Atypical gliosis	1	10	9	180	IS GTCS	Lt T/T	II	Focal encephalmalacia	Focal hypometabolism	Hypsarrhythmia, origin of left temporal (ictal phase)	VPA PDN LEV	WB
17	F	Atypical gliosis	5	24	19	400	IS Tonic	Rt hemisphere/T	I	Unilateral hemisphere atrophy	Hypometabolism of unilateral hemisphere	Variant Hypsarrhythmia, multifocal origin of right hemisphere (ictal phase)	ACTH TPM CLZ LEV VPA	PCR WB IHC

MRI, magnetic resonance imaging; PET, positron emission tomography; EEG, electroencephalograph; AEDs, anti-epileptic drugs; M, man; F, female; Lt, left; Rt, right; FR, frontal; P, parietal; O, occipital; T, temporal; IS, infantile spasm; GTCS, generalized tonic–clonic seizure; PS, partial seizure; TPM, topiramate; ACTH, adrenocorticotropic hormone; VPA, valproate; LEV, levetiracetam; LTG, lamotrigine; CLZ, clonazepam; PDN, prednisone; PCR, Real-time PCR, WB, Western Blot, IHC, immunohistochemistry (including immunofluorescence); PO, Engel's class. CD, cortical dysplasia; FCD, focal cortical dysplasia.

Table 2 Clinical and neuropathologic features of autopsy control patient samples

Case no.	Gender	Age years	Cause of death	Pathology examination	PMI (h)	Cortical Region Sampled	Application in the current study
1	M	2	Non-neurological disease (Acute heart failure)	Normal	1	Frontal	PCR WB IHC
2	F	4	Craniocerebral injury	Normal	3	Temporal	PCR WB IHC
3	M	5	Traffic accident	Normal	6	Occipital	PCR WB IHC
4	M	3	Congenital heart disease	Normal	2	Parietal	PCR WB IHC
5	F	1	Acute brain hemorrhage (pons)	Normal	1	Frontal	WB IHC
6	M	2	Traffic accident	Normal	5	Temporal	PCR WB IHC

M, Man; F, female; PMI, postmortem interval; PCR, Real-time PCR; WB, Western Blot; IHC, immunohistochemistry (including immunofluorescence).

For double immunofluorescence staining, the tissues were incubated with the primary antibody anti-human CRH (rabbit polyclonal, 1:250; Abcam), anti-human CRHR1 (goat polyclonal, 1:50; Abcam), anti-human PKC (mouse monoclonal, 1:50; Boster, Wuhan, China), respectively, mixed with anti-NF200 (mouse monoclonal, 1:100; Boster, wuhan) or anti-NF200 (rabbit polyclonal, 1:200; Abcam), anti-GFAP (mouse monoclonal, 1:400; Sigma) or anti-GFAP (rabbit monoclonal, 1:400; Abcam), anti-HLA (mouse monoclonal, 1:200, Dako, Glostrup, Denmark), or anti-HLA (rabbit monoclonal, 1:200, Abcam) overnight at 4°C. After three washes, the tissue slices were incubated with a mixture solution of FITC-conjugated antibodies and Alexa Fluor 594 antibodies (1:200, Boster, China) for 1 h at 37°C. The cell nucleolus were stained using 4′,6-diamidine-2-phenylindole (DAPI, 10 μg/mL, Beytime, China). The fluorescent slices were observed and photographed under a confocal laser scanning microscope (Leica, Nussloch, Germany).

Evaluation of Immunochemistry

All staining sections were reviewed according with the previous methods [23]: 10 digital image at 1360 × 1024 pixel resolution at 400× magnification were captured by the Olympus BX63 microscope (Olympus, Shinjuku-ku, Tokyo, Japan). We analyzed the digital images by Image Pro Plus software (IPP) (Media Cybernetics, Bethesda, MD, USA), and density mean was measured. First, we calibrated the optical density, set the area of interest through: hue, 0–39; saturation, 0–255; intensity, 0–255; then converted the image to gray image and counted the values with the data collector to collect data.

Statistical Analyses

The data are expressed as means ± SD. Statistical analyses were performed using SPSS 18.0 software (Chicago, IL, USA). Means of experimental groups were compared using independent-samples *t*-test, and the *P*-value <0.05 was considered statistically significant. The relations between variates were examined by the Spearman rank relation test.

Results

General Patient Information

The study group included 17 patients with IS, 12 males and five females. The average age at surgery was 29.4 months (range: 9–58 months), the average age of convulsion onset was 6.3 months (rang: 1–30 months), and the duration of epileptic spasm was 19.2 months (range: 8–46 months). All patients had epileptic spasms, and some patients also had partial seizures (41%), tonic seizures (24%), and generalized tonic–clonic seizure (18%). The average frequency of epileptic spasm before surgery was 304 times (range: 120–900 times) per month. The MRI scans showed cortical lesions in 15 patients, including eight cortical dysplasia and seven atrophy or encephalomalacia. The PET scans showed unifocal cortical hypometabolism in 11 patients and hemispheric hypometabolism in six patients. Fifteen of them were consistent with the MRI findings, and the other two was consistent with the

electroencephalogram (EEG) findings. The EEG showed interictal hypsarrhythmia in eight patients and variant hypsarrhythmia in the remaining nine patients (their previous EEG displayed typical hypsarrhythmia), and showed ictal focal discharge origins in 13 patients and unilateral hemispheric multifocal discharge in the other four patients. The patients had unifocal (seven cases), unilateral multilobular (six cases), and unihemispheric resection (four cases). The Engel classification was applied for assessing the surgical outcome, including 12 class I, three class II, and two class III (Table 1).

Real-time PCR and Western Blotting Analysis of CRH and CRHR1

Real-time PCR was used to determine the mRNA expressions of CRH, CRHR1 and CRHR2 in both the epileptogenic tissues of patients with IS and the control specimens. The results showed that the mRNA expressions of CRH and CRHR1 were significantly upregulated in the epileptogenic tissues of IS compared with the control group (Figure 1A,B). Western blotting showed similar results and indicated that the expressions of CRH and CRHR1 were significantly up-regulated in the epileptogenic tissues of IS compared with the control group (Figure 1C–F). But, the expression of CRHR2 was not significantly different in the epileptogenic tissues of patients with IS than in the control group (results not shown).

Immunohistochemical Analysis of CRH

H&E staining showed that the characteristics of focal cortical dysplasia type Ia (FCD Ia) included the disordered cortex and microcolumns (Figure 2A). Immunohistochemistry were used to detect the expression of CRH in the specimens from patients with IS and the controls. The results showed weak CRH immunoreactivity (IR) in neurons (Figure 2B) in the control. However, neurons displayed moderate-to-strong CRH IR (Figure 2C) in the epileptogenic tissues of IS. The density mean showed that CRH IR were significantly increased in the IS group than in the control group (Table 3). Double-labeling staining showed that CRH was co-expressed in neurons with NF200 (Figure 2D–F), but was not co-expressed in astrocytes and microglia with GFAP and HLA (Figure 2G–L).

Immunohistochemical Analysis of CRHR1

Next, we detected the expression of CRHR1 in the specimens of patients with IS and the control specimens. H&E staining showed the normal cortex displayed well-preserved cortical lamination, with the apical dendrite extending to the pial surface (Figure 3A). The immunohistochemical results showed weak-to-moderate CRHR1 IR in neurons and glial cells (Figure 3B) in the control. However, neurons and glial cells

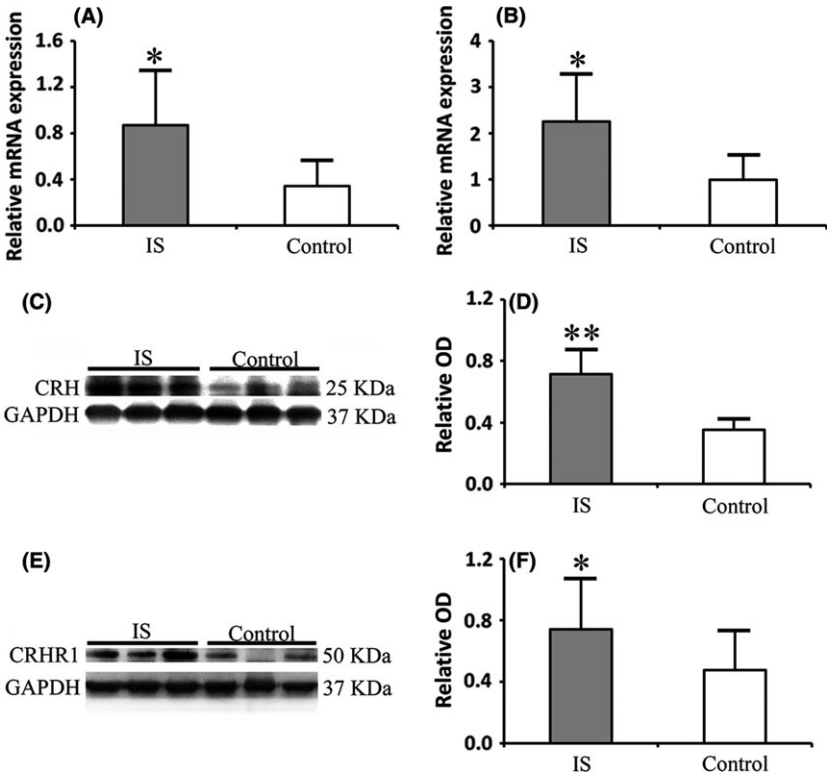

Figure 1 Real-time PCR of CRH and CRHR1mRNA expressions in control cortexes (n = 5) and infantile spasms (IS) specimens (n = 5). Messenger RNA level for CRH (**A**) and CRHR1 (**B**) was significantly upregulated in IS specimens compared to Controls. Respectively $*P = 0.037$, $*P = 0.019$, independent-samples *T* test. (**C–F**) Alteration of corticotropin-releasing hormone (CRH) and corticotropin-releasing hormone receptor 1 (CRHR1) expression in the epileptogenic tissues of IS. (**C–F**) Representative immunoblot bands (**C, E**) and densitometric analysis (**D, F**) of total homogenates from IS specimens and Controls. CRH and CRHR1 are significantly increased in the epileptogenic tissues of IS compared to Controls. Respectively $**P < 0.001$, $*P = 0.038$, independent-samples *T* test.

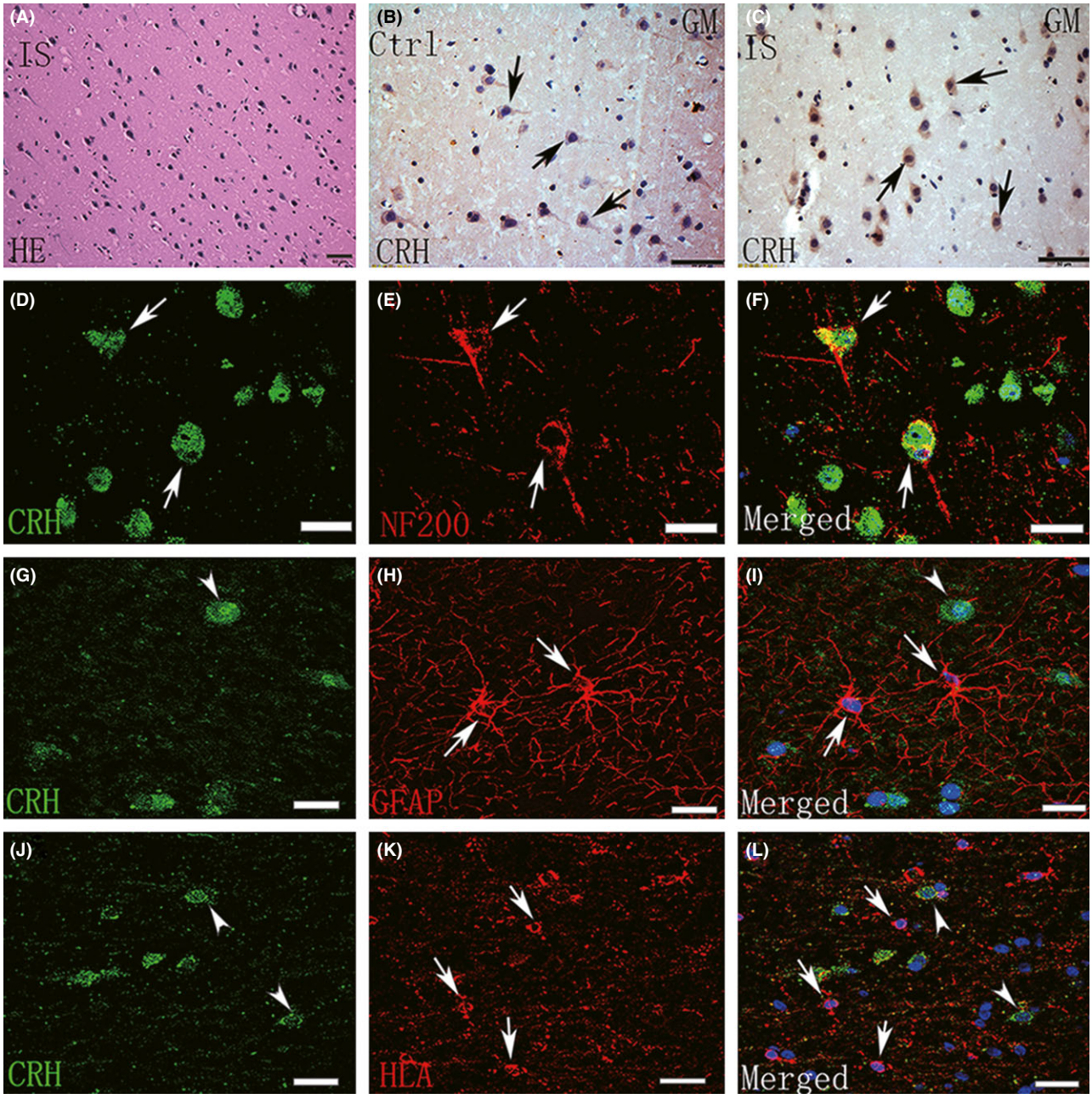

Figure 2 Representative hematoxylin and eosin staining photomicrographs of FCD Ia in infantile spasms (IS) samples. (**A**) FCD Ia samples showing cortical disorganization with the presence of microcolumns. (**B–L**) Immunoreactivity (IR) of CRH in epileptogenic tissues of IS and Controls. Weak CRH IR in neurons of normal cortex (arrows in **B**) and moderate to strong CRH IR in normal-appearing neurons of epileptogenic tissues of IS (arrows in **C**). Co-localization of CRH (green) with NF200 (red) in normal-appearing neurons (arrows in DF). No CRH positive (green) astrocytes co-localized with GFAP (red) (arrows in **G–I**), No CRH positive (green) microglia co-localized with HLA (red) (arrows in **J–L**). Scale bars, 50 μm for **A–C** panels, 25 μm for **D–L** panels.

displayed moderate-to-strong CRHR1 IR (Figure 3C) in the epileptogenic tissues of IS. The density mean showed that CRHR1 IR were significantly increased in the IS group than in the control group (Table 3). Double-labeling staining showed that CRHR1 was coexpressed in neurons with NF200 (Figure 3D–F), in astrocytes with GFAP (Figure 3G–I) and in microglia with HLA (Figure 3J–L).

PKC and PKA Expression

Then, we investigated the expressions of PKC and PKA, which are considered the important downstream factors of CRHR1. Western blotting revealed that the expression of PKC was significantly upregulated in the epileptogenic tissues of IS compared with the control group (Figure 4A,B), but PKA expression were not

Table 3 Density Mean of CRH, CRHR1, and PKC in Control and IS

	Control (n = 6)	IS (n = 10)
CRH	0.0063 ± 0.0024	0.0140 ± 0.0027**
CRHR1	0.0061 ± 0.0011	0.0120 ± 0.0025**
PKC	0.0069 ± 0.0024	0.0127 ± 0.0022**

Control, normal control; IS, infantile spasms; CRH, Corticotrophin-releasing hormone; CRHR1, Corticotrophin-releasing hormone receptor 1; PKC, protein kinase C. Data are expressed as mean ± SD. The density mean of CRH, CRHR1 and PKC determined by Image Pro-Plus. $^{**}P < 0.001$, IS versus Control, independent-samples *t*-test.

different in the epileptogenic tissues of patients with IS than in the control group (results not shown). H&E staining showed atypical gliosis was characterized as an abnormal increase in the number of glial cells (Figure 4A). Immunohistochemistry demonstrated that neurons and glial cells displayed weak-to-moderate PKC IR (Figure 4D) in the controls and moderate-to-strong PKC IR in the epileptogenic tissues of patients with IS (Figure 4E). The density mean showed that PKC IR was higher in the epileptogenic tissues of patients with IS than in the control group (Table 3). Double-labeling showed that PKC was coexpressed in neurons with NF200 (Figure 4F–H), in astrocytes with GFAP (Figure 4I–K) and in microglia with HLA (Figure 4L–N).

The Correlation between CRH, CRHR1, PKC, and the Epileptic Spasms

Furthermore, we analyzed the correlation between CRH, CRHR1, PKC, and the epileptic spasm**s**. The expression levels of both CRH and CRHR1 were positively correlated with the frequency of epileptic spasms (CRH, $r = 0.671$, $P < 0.05$; CRHR1, $r = 0.689$, $P < 0.05$) (Figure 5A,B), but were not significantly correlated with other clinical variables. In addition, the expression levels of PKC were positively correlated with the frequency of epileptic spasms ($r = 0.72$, $P < 0.05$) (Figure 5C), and the expression levels of CRHR1 ($r = 0.855$, $P < 0.01$) (Figure 5D), but were not correlated with other clinical variables.

Discussion

In this study, we found that the expressions of CRH and CRHR1 were significantly higher in the epileptogenic tissues of patients with IS than in the control group. CRH was distributed mainly in neurons, while CRHR1 was distributed in neurons, astrocytes, and microglia. There were positive correlation between the expression levels of CRH and CRHR1 and the frequency of epileptic spasms. In addition, PKC was highly expressed in the epileptogenic tissues of IS and was positively correlated with the CRHR1 expression levels and the frequency of epileptic spasms. The above results suggest that the CRH-CRHR1-PKC signaling pathway maybe participate in the epileptogenesis of IS.

Excessive Expression of CRH in IS

Previous study indicated both ACTH and cortisol levels in the cerebrospinal fluid of infantile patients with IS are downregulated and ACTH played an anticonvulsant role by inhibiting CRH expression in the amygdala of immature rat [2–4]. Animal studies have shown that the seizure provoked by CRH was age-specific in the developing rat; for example, the required doses for adult rats were 200 times bigger than those required for seizure generation in rats at second postnatal weeks [24,25]. Several studies also showed that CRH increased the excitability of multiple subareas of the hippocampus [24,26]. One study demonstrated that the expression of CRH was elevated in autopsy brain tissues obtained from pediatric patients (5–15 years) with generalized epilepsy compared with the control [12]. Therefore, Brunson et al. speculated that CRH may target a specific cortex to trigger the epileptogenesis of human IS and proposed the hypothesis that excessive CRH in the brain is one of etiology of IS [8,27]. However, there was no direct evidence of CRH in the brain tissues of human IS to illustrate this theory. In this study, we first found that CRH was significantly higher in the epileptogenic tissues of infantile patients with IS than in the control group. Furthermore, its receptor CRHR1 and downstream PKC signaling were activated and associated with the frequency of epileptic spasms, suggesting that excessive CRH in a specific cortex might participate in the epileptogenesis of IS.

Enhanced Expression of CRHR1 in IS

CRHR1 plays a major role due to its high affinity and specific distribution in brain [9,10]. Limbic seizures induced by CRH in the immature rats can be blocked by CRHR1 antagonist, suggesting that the convulsant effect of CRH in the immature brain may be mediated by CRHR1 [11]. Our study further indicated that CRHR1 may be an important receptor that is involved in the role of CRH in the epileptogenic tissues of infantile patients with IS. CRH has been confirmed to induce CRHR1 expression in the cell membrane in the immature brain [13], whereas this effect was decreased in adult brain [13,28]. In our specimens from young children (under 5 years old), the expressions of CRH and CRHR1 were simultaneously upregulated and that were correlated with the frequency of epileptic spasms. The results suggested that CRH might promote the expression of CRHR1 in immature brains, which participating in the age-specific epileptogenesis of IS during a specific developmental stage of the brain.

Changed of PKC not PKA in IS

Electrophysiological studies of hippocampal pyramidal neurons showed that PKC is an important factor in the functional modifications with epileptic susceptibility and participated in the mechanisms of pathogenesis and maintenance of epilepsy; moreover, excitatory synaptic transmission was increased with PKC activation and the excitability of postsynaptic neurons was upregulated [15,29,30]. Other studies have suggested that PKC activation increased the expression of NMDA receptor and reduced the neuropeptide Y inhibition of glutamate release, thereby exerting seizure-promoting and potentially epileptogenic effects [31,32]. Further study suggested that PKC activation upregulated the excitatory synaptic transmission in human dentate granule cells [33]. Some previous studies indicated CRHR1-PKC signal pathway involves in neuronal excitability [34–36]. CRHR1-PKC also involved in increased firing rate of

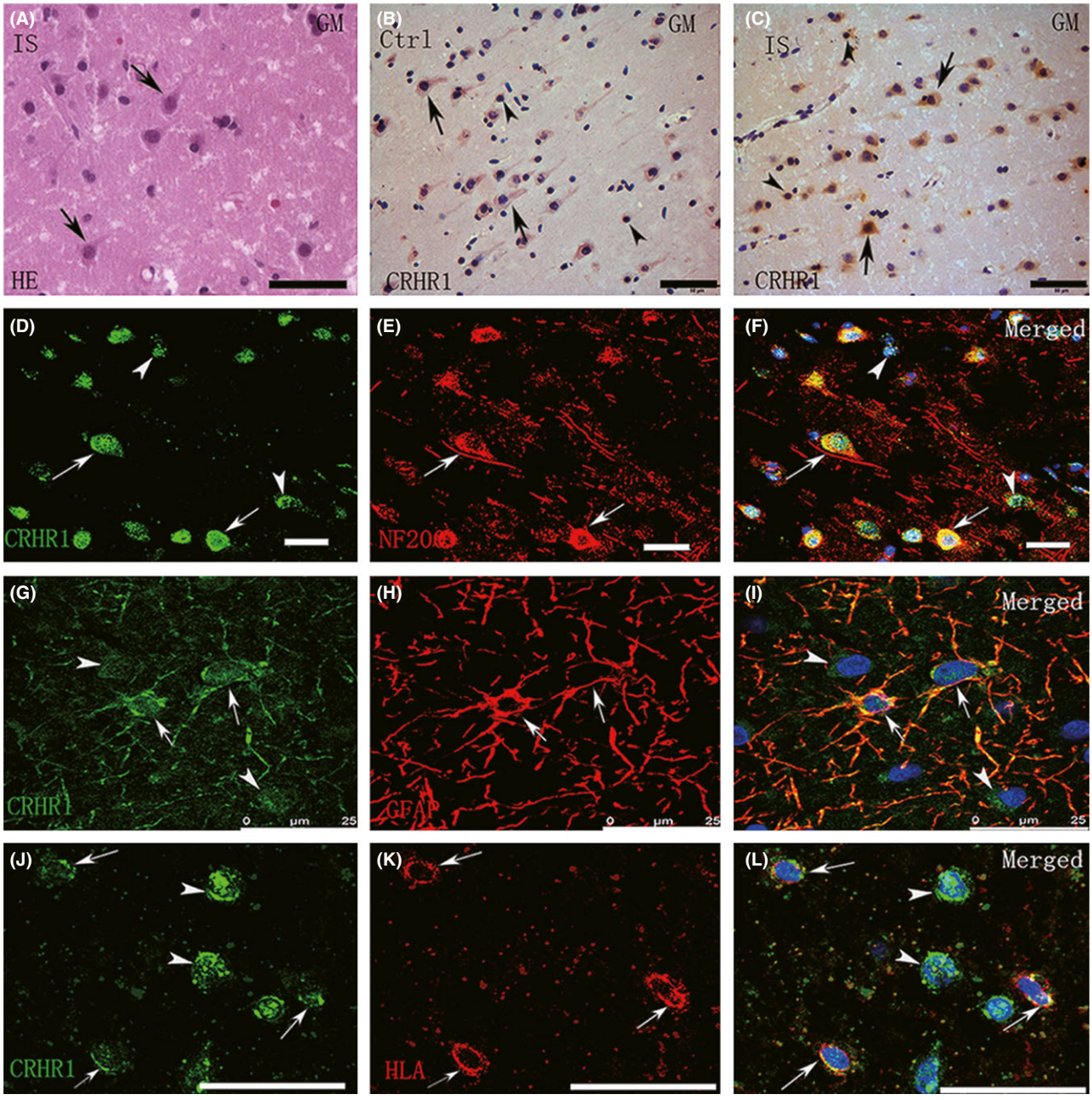

Figure 3 Representative hematoxylin and eosin staining photomicrographs of the normal-appearing cortexes in infantile spasms (IS) samples (**A**). (**B–L**) Immunoreactivity (IR) of CRHR1 in epileptogenic tissues of IS and Controls. Weak to moderate CRHR1 IR in neurons (arrows in **B**) and glial cells (head arrows in **B**) in Control, Moderate to strong CRHR1 IR in normal-appearing neurons (arrows in **C**) and glia cells (head arrows in **C**) in epileptogenic tissues of IS. Double-labeled immunofluorescence shows co-localization of CRHR1 (green) with NF200 (red) in normal-appearing neurons (arrows in **D–F**). CRHR1 positive (green) astrocytes co-localized with GFAP (red) (arrows in **G–I**), CRHR1 positive (green) microglia (arrows) co-localized with HLA (red) (arrows in **J–L**). Scale bars, 50 μm for **A–C** panels, 25 μm for **D–L** panels, 10 μm for **L–N** panels.

dopamine neuron in ventral tegmental area [37]. In this study, the results showed that PKC was increased in the epileptogenic tissues of patients with IS and was positively correlated with the frequency of epileptic spasms and CRHR1 expression. Therefore, we speculated that the intracellular CRH-CRHR1-PKC signal pathway may be involved in the epileptogenesis of IS, but requires further investigation.

CHR-CRHR1-PKC Signal Pathway in Neuroglial Cells

CRH may induce the proliferation of astrocytes and increase the intracellular Ca2+ concentration via CRH Receptor [38,39], which induced the release of Ca2+-dependent glutamate and other glio-transmitters from glial cells [40], and enhanced the excitability of

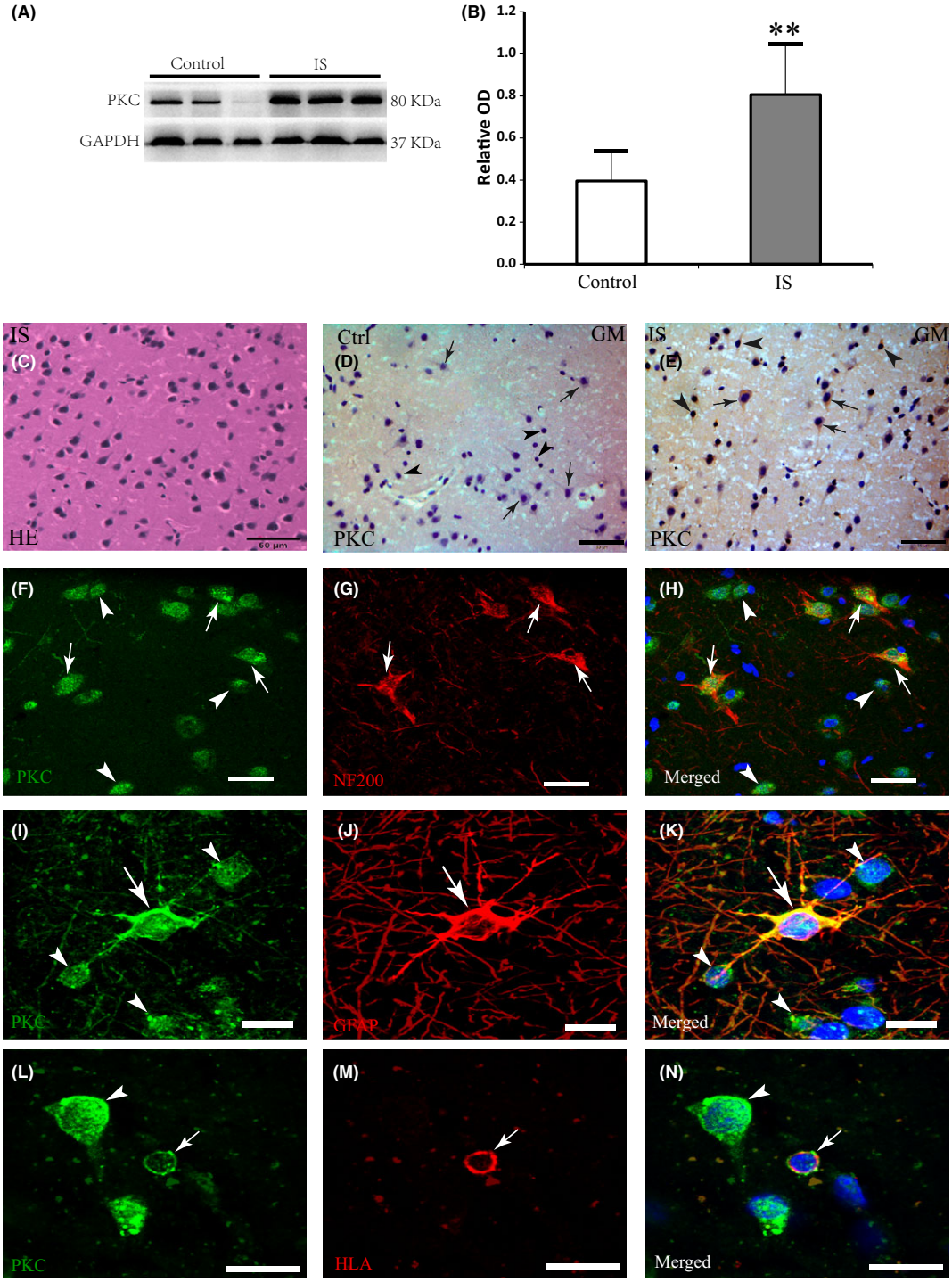

Figure 4 Alteration of Protein Kinase C (PKC) in epileptogenic tissues of infantile spasms (IS). (**A**, **B**) Representative immunoblot bands (**A**) and densitometric analysis (**B**) of total homogenates from control cortexes and IS specimens. PKC are significantly increased in epileptogenic tissues of IS compared to Controls. $^{**}P < 0.001$, independent-samples *T* test. (**C**–**N**) Cell specific distribution of PKC in Controls and IS specimens. Representative hematoxylin and eosin staining photomicrographs of atypical gliosis in IS samples (**C**). Weak to moderate PKC Immunoreactivity (IR) in neurons (arrows in D) and glia cells (headarrows in **D**) of control cortex, moderate to strong PKC IR in normal-appearing neurons (arrows in **E**) and glia cells (headarrows in **E**) of epileptogenic tissues of IS. Double-labeled immunofluorescence shows co-localization of PKC (green) with NF200 (red) in normal-appearing neurons (arrows in **F**–**H**). PKC positive (green) astrocytes co-localized with GFAP (red) (arrows in **I**–**K**), PKC positive (green) microglia (arrows) colocalized with HLA (red) (arrows in **L**–**N**), neuron do not co-localized with HLA(red) (arrowhead in **L, N**). Scale bars, 50 μm for **C**–**E** panels, 25 μm for **F**–**H** panels, 10 μm for **I**–**N** panels.

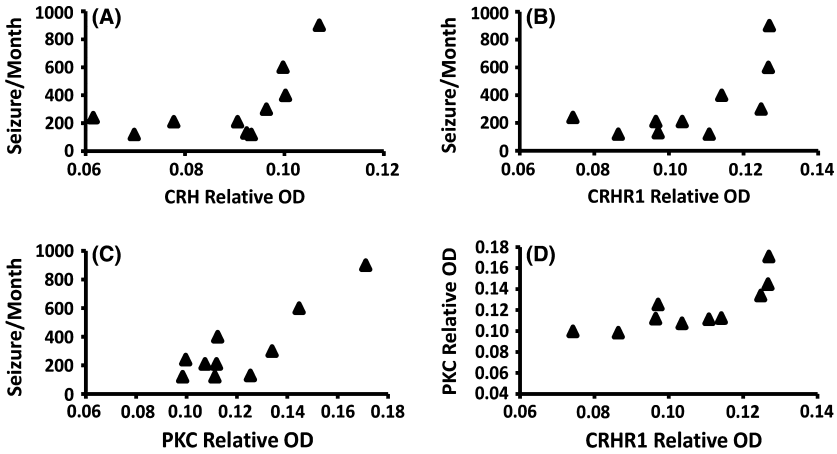

Figure 5 (**A–C**) Correlation between the expression of CRH, CRHR1 and PKC and the frequency of epileptic spasm of IS. Scatter plot showing the significant positive correlation between the expression (relative optical density [OD]) of CRH, CRHR1 and PKC and seizure frequency (seizures per month) of IS. Spearman rank correlation coefficient: CRH: $r = 0.671$, $P < 0.05$; CRHR1, $r = 0.689$, $P < 0.05$; PKC, $r = 0.72$, $P < 0.05$. (**D**) Correlation between the expression of CRHR1 and PKC in IS. Scatter plot showing the significant positive correlation between the expression (relative optical density [OD]) of PKC and the expression of CRHR1. Spearman rank correlation coefficient $r = 0.855$, $P < 0.01$.

neurons. CRH was found to regulate the neuroinflammation by inducing the apoptosis of primary microglia cells in mouse forebrain [41]. The combination of CRH and CRHR1 mediated the secretion of IL-18 from microglia via the reactive oxygen intermediate (ROI) signaling pathway [42], and promoted the expressions of TNF-α and Fas-L [43,44]. Correlations between inflammation and epilepsy have been confirmed by many studies [45]. In the present study, immunohistochemical and immunofluorescence assays demonstrated that CRH was mainly distributed in neurons, and its receptor CRHR1 and downstream molecule PKC were distributed in neurons, astrocytes, and microglia. Thus, CRH might come from mainly neurons and be involved in the epileptogenesis of IS through the responses of neurons, astrocytes, and microglia.

Conclusions

On the whole, we first observed that CRH, CRHR1, and the downstream factor PKC were highly expressed in the epileptogenic tissues of human patients with IS and were associated with the frequency of epileptic spasms. These results suggest a potential epileptogenic role for CRH signaling pathway in IS, but further studies are needed to confirm the findings.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Sorel L, Dusaucy-Bauloye A. Findings in 21 cases of Gibbs' hypsarrhythmia; spectacular effectiveness of ACTH. *Acta Neurol Psychiatr Belg* 1958;**58**:130–141.
2. Baram TZ, Mitchell WG, Hanson RA, Snead OC 3rd, Horton EJ. Cerebrospinal fluid corticotropin and cortisol are reduced in infantile spasms. *Pediatr Neurol* 1995;**13**:108–110.
3. Baram TZ, Hirsch E, Snead OC 3rd, Schultz L. Corticotropin-releasing hormone-induced seizures in infant rats originate in the amygdala. *Ann Neurol* 1992;**31**:488–494.
4. Brunson KL, Khan N, Eghbal-Ahmadi M, Baram TZ. Corticotropin (ACTH) acts directly on amygdala neurons to down-regulate corticotropin-releasing hormone gene expression. *Ann Neurol* 2001;**49**:304–312.
5. Ehlers CL, Henriksen SJ, Wang M, Rivier J, Vale W, Bloom FE. Corticotropin releasing factor produces increases in brain excitability and convulsive seizures in rats. *Brain Res* 1983;**278**:332–336.
6. Marrosu F, Fratta W, Carcangiu P, Giagheddu M, Gessa GL. Localized epileptiform activity induced by murine CRF in rats. *Epilepsia* 1988;**29**:369–373.
7. Baram TZ, Schultz L. Corticotropin-releasing hormone is a rapid and potent convulsant in the infant rat. *Brain Res Dev Brain Res* 1991;**61**:97–101.
8. Brunson KL, Eghbal-Ahmadi M, Baram TZ. How do the many etiologies of West syndrome lead to excitability and seizures? The corticotropin releasing hormone excess hypothesis. *Brain Dev* 2001;**23**:533–538.
9. Lovenberg TW, Liaw CW, Grigoriadis DE, et al. Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. *Proc Natl Acad Sci U S A* 1995;**92**:836–840.
10. Reul JMHM, Holsboer F. Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. *Curr Opin Pharmacol* 2002;**2**:23–33.
11. Baram TZ, Chalmers DT, Chen C, Koutsoukos Y, De Souza EB. The CRF1 receptor mediates the excitatory actions of corticotropin releasing factor (CRF) in the developing rat brain: In vivo evidence using a novel, selective, non-peptide CRF receptor antagonist. *Brain Res* 1997;**770**:89–95.
12. Wang W, Dow KE, Fraser DD. Elevated corticotropin releasing hormone/corticotropin releasing hormone-R1 expression in postmortem brain obtained from children with generalized epilepsy. *Ann Neurol* 2001;**50**:404–409.
13. Brunson KL, Grigoriadis DE, Lorang MT, Baram TZ. Corticotropin-releasing hormone (CRH) downregulates the function of its receptor (CRF1) and induces CRF1 expression in hippocampal and cortical regions of the immature rat brain. *Exp Neurol* 2002;**176**:75–86.
14. Grammatopoulos DK, Chrousos GP. Functional characteristics of CRH receptors and potential clinical applications of CRH-receptor antagonists. *Trends Endocrinol Metab* 2002;**13**:436–444.
15. Grabaliskas G, Chapman H, Wheal HV. Role of protein kinase C in modulation of excitability of CA1 pyramidal neurons in the rat. *Neuroscience* 2006;**139**:1301–1313.
16. Pellock JM, Hrachovy R, Shinnar S, et al. Infantile spasms: A U.S. consensus report. *Epilepsia* 2010;**51**: 2175–2189.

17. Nelson GR. Management of infantile spasms. *Transl Pediatr* 2015;**4**:260–270.
18. Chugani HT, Asano E, Sood S. Infantile spasms: Who are the ideal surgical candidates? *Epilepsia* 2010;**51**:94–96.
19. Asano E, Chugani DC, Juhasz C, Muzik O, Chugani HT. Surgical treatment of West syndrome. *Brain Dev* 2001;**23**:668–676.
20. Hynd MR, Lewohl JM, Scott HL, Dodd PR. Biochemical and molecular studies using human autopsy brain tissue. *J Neurochem* 2003;**85**:543–562.
21. Preece P, Cairns NJ. Quantifying mRNA in postmortem human brain: Influence of gender, age at death, postmortem interval, brain pH, agonal state and inter-lobe mRNA variance. *Brain Res Mol Brain Res* 2003;**118**:60–71.
22. Perry EK, Perry RH, Tomlinson BE. The influence of agonal status on some neurochemical activities of postmortem human brain tissue. *Neurosci Lett* 1982;**29**:303–307.
23. Wang CJ, Zhou ZG, Holmqvist A, et al. Survivin expression quantified by Image Pro-Plus compared with visual assessment. *Appl Immunohistochem Mol Morphol* 2009;**17**:530–535.
24. Hollrigel GS, Chen K, Baram TZ, Soltesz I. The pro-convulsant actions of corticotropin-releasing hormone in the hippocampus of infant rats. *Neuroscience* 1998;**84**: 71–79.
25. Baram TZ, Hatalski CG. Neuropeptide-mediated excitability: A key triggering mechanism for seizure generation in the developing brain. *Trends Neurosci* 1998;**21**:471–476.
26. Aldenhoff JB, Gruol DL, Rivier J, Vale W, Siggins GR. Corticotropin releasing factor decreases postburst hyperpolarizations and excites hippocampal neurons. *Science* 1983;**221**:875–877.
27. Galanopoulou AS. Basic mechanisms of catastrophic epilepsy – Overview from animal models. *Brain Dev* 2013;**35**:748–756.
28. Mansi JA, Rivest S, Drolet G. Regulation of corticotropin-releasing factor type 1 (CRF1) receptor messenger ribonucleic acid in the paraventricular nucleus of rat hypothalamus by exogenous CRF. *Endocrinology* 1996;**137**:4619–4629.
29. Shin J, Shen F, Huguenard J. PKC and polyamine modulation of GluR2-deficient AMPA receptors in immature neocortical pyramidal neurons of the rat. *J Physiol* 2007;**581**:679–691.
30. Hu GY, Hvalby O, Walaas SI, et al. Protein kinase C injection into hippocampal pyramidal cells elicits features of long term potentiation. *Nature* 1987;**328**:426–429.
31. Lan JY, Skeberdis VA, Jover T, et al. Protein kinase C modulates NMDA receptor trafficking and gating. *Nat Neurosci* 2001;**4**:382–390.
32. Silva AP, Lourenco J, Xapelli S, et al. Protein kinase C activity blocks neuropeptide Y-mediated inhibition of glutamate release and contributes to excitability of the hippocampus in status epilepticus. *FASEB J* 2007;**21**: 671–681.
33. Chen HX, Roper SN. PKA and PKC enhance excitatory synaptic transmission in human dentate gyrus. *J Neurophysiol* 2003;**89**:2482–2488.
34. Blank T, Nijholt I, Grammatopoulos DK, Randeva HS, Hillhouse EW, Spiess J. Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: Role in neuronal excitability and associative learning. *J Neurosci* 2003;**23**:700–707.
35. Sheng H, Xu Y, Chen Y, Zhang Y, Ni X. Corticotropin-releasing hormone stimulates mitotic kinesin-like protein 1 expression via a PLC/PKC-dependent signaling pathway in hippocampal neurons. *Mol Cell Endocrinol* 2012;**362**:157–164.
36. Tan H, Zhong P, Yan Z. Corticotropin-releasing factor and acute stress prolongs serotonergic regulation of GABA transmission in prefrontal cortical pyramidal neurons. *J Neurosci* 2004;**24**:5000–5008.
37. Wanat MJ, Hopf FW, Stuber GD, Phillips PE, Bonci A. Corticotropin-releasing factor increases mouse ventral tegmental area dopamine neuron firing through a protein kinase C-dependent enhancement of Ih. *J Physiol* 2008;**586**:2157–2170.
38. Takuma K, Matsuda T, Yoshikawa T, et al. Corticotropin-releasing factor stimulates Ca2 + influx in cultured rat astrocytes. *Biochem Biophys Res Commun* 1994;**199**: 1103–1107.
39. Ha BK, Bishop GA, King JS, Burry RW. Corticotropin releasing factor induces proliferation of cerebellar astrocytes. *J Neurosci Res* 2000;**62**:789–798.
40. Takemiya T, Yamagata K. Intercellular signaling pathway among endothelia, astrocytes and neurons in excitatory neuronal damage. *Int J Mol Sci* 2013;**14**:8345–8357.
41. Ock J, Lee H, Kim S, et al. Induction of microglial apoptosis by corticotropin-releasing hormone. *J Neurochem* 2006;**98**:962–972.
42. Yang Y, Hahm E, Kim Y, et al. Regulation of IL-18 expression by CRH in mouse microglial cells. *Immunol Lett* 2005;**98**:291–296.
43. Wang W, Ji P, Riopelle RJ, Dow KE. Functional expression of corticotropin-releasing hormone (CRH) receptor 1 in cultured rat microglia. *J Neurochem* 2002;**80**:287–294.
44. Wang W, Ji P, Dow KE. Corticotropin-releasing hormone induces proliferation and TNF-alpha release in cultured rat microglia via MAP kinase signalling pathways. *J Neurochem* 2003;**84**:189–195.
45. Vezzani A, French J, Bartfai T, Baram TZ. The role of inflammation in epilepsy. *Nat Rev Neurol* 2011;**7**:31–40.

