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·Original Article·

Early hypoactivity of hippocampal rhythms during epileptogenesis after prolonged febrile seizures in freely-moving rats

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ABSTRACT

Prospective and experimental studies have shown that individuals with early-life complex/prolonged febrile seizures (FSs) have a high incidence of temporal lobe epilepsy during adulthood, revealing a close relationship between FSs and epilepsy. However, little is known about how epileptogenesis develops after FSs. The present study was designed to investigate acquired seizure susceptibility and analyze local field potentials during the latent period after FSs. We found that the seizure susceptibility decreased in 35-day-old (P35) FS rats but increased in P60 FS rats. Consistently, hippocampal electroencephalogram (EEG) power in every band was decreased at P35 but increased at P60 in FS rats. Our results provide direct evidence for hypoactivity but not hyperactivity during the early phase of the latent period, displaying a broad decrease in hippocampal rhythms. These characteristic EEG changes can be a useful biomarker for the early diagnosis of epileptogenesis induced by FSs.

Keywords: febrile seizure; seizure susceptibility; epilepsy; hippocampal rhythms

INTRODUCTION

Febrile seizures (FSs) are the most common seizure type during childhood[1]. Although most of them are simple and apparently benign, children with complex FSs, repeated or prolonged FSs (>30 min) show a high incidence of temporal lobe epilepsy and sclerosis in later life[2, 3]. Recent studies of FSs induced by hyperthermia in immature rodents have shown that early-life FSs lead to a reduced seizure threshold[4] and even spontaneous recurrent seizures during adulthood[5]. These results reveal a close relationship between early-life FSs and epilepsy[6]. However, to date, little is known about how a normal brain develops into the epileptic state after FSs in early life, i.e., epileptogenesis. This hampers diagnosis, the development of biomarkers, and the prevention of epileptogenesis following FSs.

The electroencephalogram (EEG) during seizure activity has been the gold standard in the diagnosis of epilepsy for several decades[7]. However, there is still no reliable biomarker for the diagnosis of epileptogenesis. In recent years, some potential EEG biomarkers have been explored in adult animals[8], such as decreased θ and α oscillations during the latent period after pilocarpine-induced status epilepticus[9, 10]. In addition, since the EEG pattern in adolescents differs from that in adults[11-13], the

EEG changes/biomarkers in adolescents ought to be studied independently.

Thus, in order to search for reliable EEG biomarkers, we focused on the changes of hippocampal EEG during the period of development after infantile FSs induced by hyperthermia by analyzing the local field potential.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were individually housed under a 12-h light/dark cycle (lights on 08:00-20:00) with access to water and food *ad libitum*. Experiments were carried out between 10:00 and 17:00. All experiments were approved by the Zhejiang University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize the number of animals used and their suffering.

Generation of Prolonged Febrile Seizures

Prolonged FSs were induced in rats on postnatal day 8 (P8) as described previously[14]. The body temperature of the pups was raised in a hyperthermia chamber (44 ± 0.5°C) until a seizure was induced. The body temperature at the onset of behavioral seizures was measured by a rectal probe (Temperature Control machine, Bowdoinham, ME). The behavioral characteristics of seizures included sudden movement arrest followed by facial automatisms (chewing), forelimb clonus, and tonic flexion of the body that was often associated with a loss of postural control. After 55 min of hyperthermia, the pups were removed from the chamber. Pups with observed seizures were removed to a cool surface for 2 min and then returned to the chamber. Fifty-five minutes of hyperthermia did not result in dehydration. The weight decreased 3.2 ± 0.5 g, the seizure latency was 27.78 ± 1.1 min, and the seizure threshold was 41.8 ± 0.1°C (n = 8). Littermates of the experimental rats were assigned to the control group. They were removed from their cages for the same duration, and their core temperatures remained within the normal range .

Maximal Electroshock (MES)-induced Seizures

When the animals reached postnatal days 35, 45, and 60 (Fig. 1A), electroshocks were delivered using a Rodent Shocker (Hugo Sachs Elektronik, March-Hugstetten, Germany). Electrodes moistened with saline were clipped to the ears. The convulsion patterns were assigned stages based on the extent of the spread of tonic extension[15] as follows: 0, absence of forelimb extension; 1, complete forelimb extension without hindlimb extension; 2, complete forelimb extension with partial hindlimb extension; and 3, complete fore- and hindlimb extension (with hindlimbs fully extended parallel to the tail).

EEG Recording *in vivo*

Once FS rats and littermate controls reached the appropriate ages, EEG recording was performed [P35, P45,

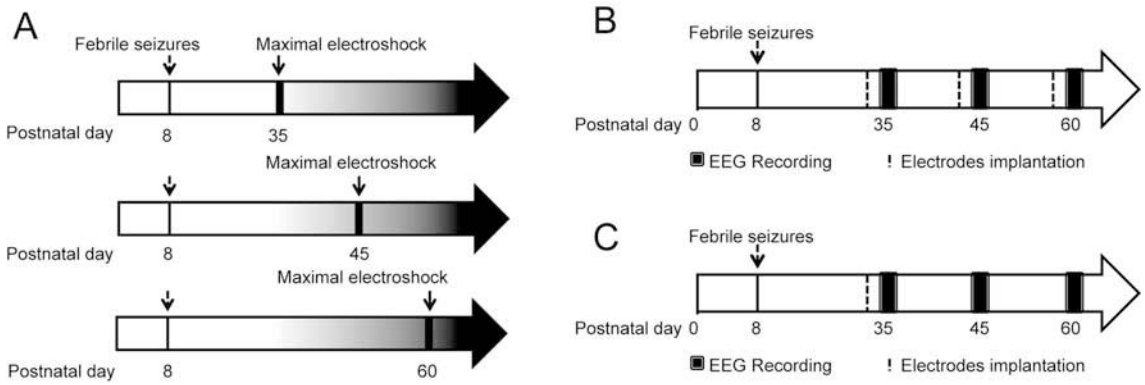

Fig. 1. Experiment design. (A) Schematic of the MES-induced seizure protocols in rats at P35, P45, and P60 (from top to bottom). (B) Study design of the EEG recordings. Surgery was performed 7 days before EEG recording at P26, P36, and P51. (C) Study design of the chronic EEG recordings. Surgery was performed 7 days before EEG recording at P26. The recordings were performed for 5 consecutive days around P35, P45, and P60 (P33–37, P43–47, and P58–62; at least 15 min every day).

and P60 for Experiment 1 (Fig. 1B); P35 for Experiment 2 (Fig. 1C)]. Seven days before the recording, electrodes were implanted into the brain. Briefly, the rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.), and mounted in a stereotaxic apparatus (512600, Stoelting, Wood Dale, IL). Twisted-wire bipolar electrodes with 0.5-mm vertical tip separation[16] were implanted in the right ventral hippocampus (immature brain, AP: −5.2 mm, L: −4.9 mm, V: −6 mm; with reference to Bregma according to the developing rat brain atlas of Sherwood and Timiras[17]; adult brain, AP: −5.3 mm, L: −5 mm, V: −6 mm with reference to Bregma according to the atlas of Paxinos and Watson[18]), and a ground electrode was placed in the cerebellum. The assembly was anchored with dental cement and three stainless-steel screws.

In Experiment 1 (Fig. 1B), EEG recordings in rats aged around P35, P45, and P60 were performed for 5 consecutive days (P33–37, P43–47, and P58–62; at least 15 min each day). In Experiment 2 (Fig. 1C), EEG recordings in rats aged around P35 were performed for 5 consecutive days around P35 (P33–37), P45 (P43–47), and P60 (P58–62), at least 15 min each day.

EEG was recorded as described previously[19]. Raw EEG signals in a unipolar setting (~5 min long) were recorded with band-pass filters spanning DC to 200 Hz and sampled at 1000 Hz. Recorded EEG signals were analyzed offline using Scan 4.5 (Neuroscan, Compumedics Ltd, Melbourne, Australia). Each entire EEG time-series was digitally band-pass filtered from 0.3 to 100 Hz, then divided into consecutive 4-s epochs (4096 points). EEG epochs with artifacts were rejected by visual inspection and finally 50 artifact-free epochs were selected for spectral analysis by random sorting. Spectral analysis using the fast Fourier transform (FFT) was run on each 4-s epoch with a Hanning window to avoid edge effects. The FFT output provided the total power for each 4-s epoch with a frequency resolution of 0.244 Hz between zero and 100 Hz. These 0.244-Hz frequency bins were subsequently averaged within 6 frequency bands: δ (delta, 0.5–4 Hz), θ (theta, 4–8 Hz), α (alpha, 8–12 Hz), β (beta, 12–30 Hz), γ (gamma, 30–100 Hz), and total power (0–100 Hz).

Histology and Neuronal Counts

Rats of different ages were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde. Brains were removed, post-fixed in paraformaldehyde overnight at 4°C, dehydrated in 30% sucrose for 5–7 days, and serially sectioned on a freezing microtome (16 μm, coronal, Leica CM3050 S, Heidelberger, Nussloch, Germany). Mounted sections were stained with toluidine blue, and neurons were counted in the CA1 and CA3 regions of the hippocampus on 5 consecutive sections, by an investigator unaware of the experimental group. Neurons were included when the nucleus was clearly identifiable and located >50% within the selected area.

Immunohistochemistry

Brain sections (16 μm) were pretreated with 3% Triton X-100 in PBS and blocked with normal donkey serum. Then they were incubated with rabbit anti-GAD65/67 (1:300, Millipore, Merck KGaA, Germany) overnight at 4°C, followed by Alexa-488-conjugated secondary antibody for 2 h at room temperature. Neurons were subsequently stained using Neuro Trace Nissl stain (1:400, 30 min at room temperature; Life Technologies, New York). The sections were cover-slipped for microscopic analysis. Digital images were photographed using an Olympus confocal microscope. The percentage of the neuronal circumference covered by inhibitory synapses was calculated by dividing the length of GAD 65/67 puncta around the neuronal soma by the neuronal circumference[20].

Statistical Analysis

The two-tailed Student's *t*-test was applied to compare the seizure stages and power spectra of the EEG between groups. Analysis of the different powers in control and FS rats was performed using two-way analysis of variance (ANOVA) with the Bonferroni post-test. Comparisons of the power spectra among different ages were made using one-way ANOVA followed by Tukey's test. Data are presented as mean ± SEM. Statistical analysis was carried out using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). For all analyses, the tests were two-sided and P <0.05 was considered statistically significant.

RESULTS

Susceptibility to MES-induced Seizures after FSs

All the animals that had experienced infantile FSs were divided into three groups (P35, P45, and P60) with their

littermate controls, and were subjected to MES (Fig. 1A). To avoid a "ceiling effect", we used different current intensities according to age. The 35 mA stimulation for FS rats at P35 induced lower seizure stages than age-matched controls and none of them reached stage 3 with complete fore- and hind-limb extension (Fig. 2A and D). FS rats at P45, when stimulated with 40 mA, showed MES-induced seizure stages similar to control littermates (Fig. 2B), but a higher percentage of stage 3 (Fig. 2E). Adult (P60) FS rats showed higher seizure stages and a higher percentage of stage 3 than controls (current intensity, 45 mA; Fig. 2C and F). Thus, the FS rats showed an age-dependent susceptibility to MES-induced seizures: lower at P35 and higher at P60 than in age-matched littermate controls.

Hippocampal EEG Power Decreased around P35 in FS Rats

EEG recordings showed that the power in the total band (1–100 Hz) was decreased in FS rats around P35 (Fig. 3A and B) compared with age-matched controls, while there was no significant difference between P45 FS rats and their age-matched controls (Fig. 3C and D). In P60 FS rats, the total band power was greater than in age-matched controls (Fig. 3E and F).

Moreover, further analysis of EEGs showed that the power in the δ, θ, α, and β bands was much lower in FS rats around P35 (Fig. 4A and B). There was no significant difference in any frequency band between P45 FS rats and their age-matched controls (Fig. 4C and D). The power in θ, α, β and γ bands was much higher in P60 FS rats than in age-matched controls (Fig. 4E and F).

Early-phase Decrease in Hippocampal EEG Power after FSs

To further verify the changes of power spectra in FS rats, we recorded hippocampal EEG chronically in FS rats and age-matched controls from P35 to P60 (Experiment 2, Fig. 1C). EEG analysis showed that in controls, there was no

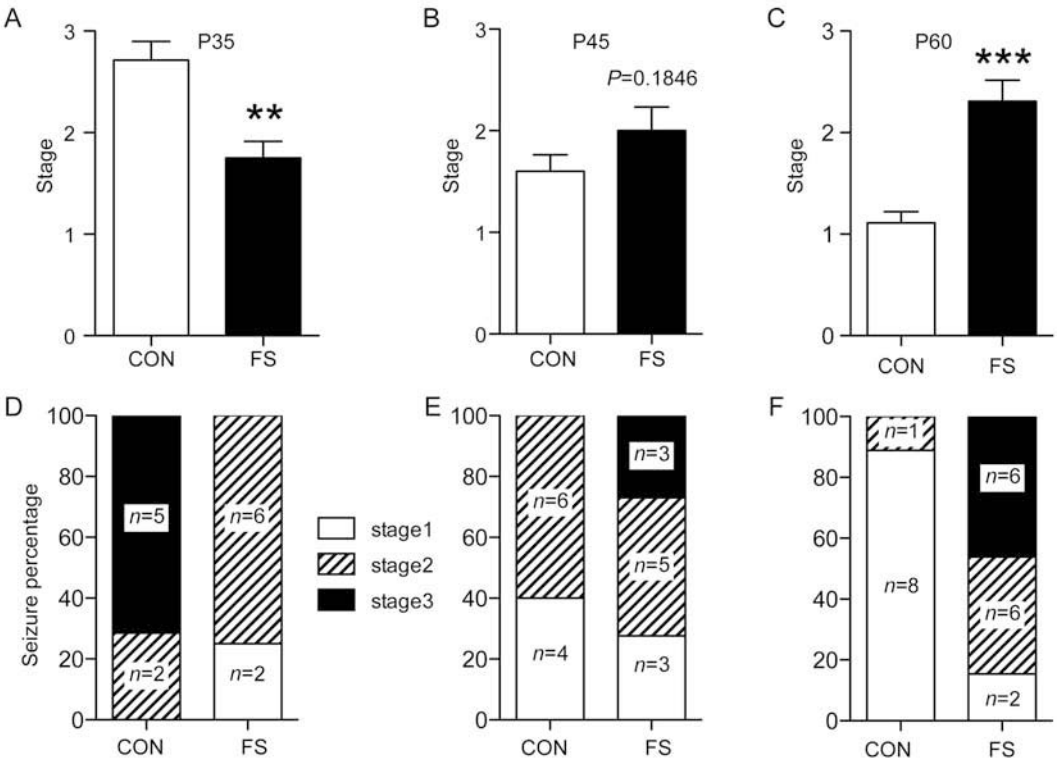

Fig. 2. MES-induced seizure susceptibility after infantile FSs in rats. (A–C) MES-induced seizure stages at P35, P45, and P60. P35 groups, *n* = 7 in control and *n* = 8 in FS; P45 groups, *n* =10 in control and *n* =11 in FS; P60 groups, *n* = 9 in control and *n* =14 in FS. (D–F) Seizure stage distribution in rats with MES-induced seizures at P35, P45, and P60. ***P* <0.01, ****P* <0.001 *vs* control group (two-tailed Student's *t*-test).

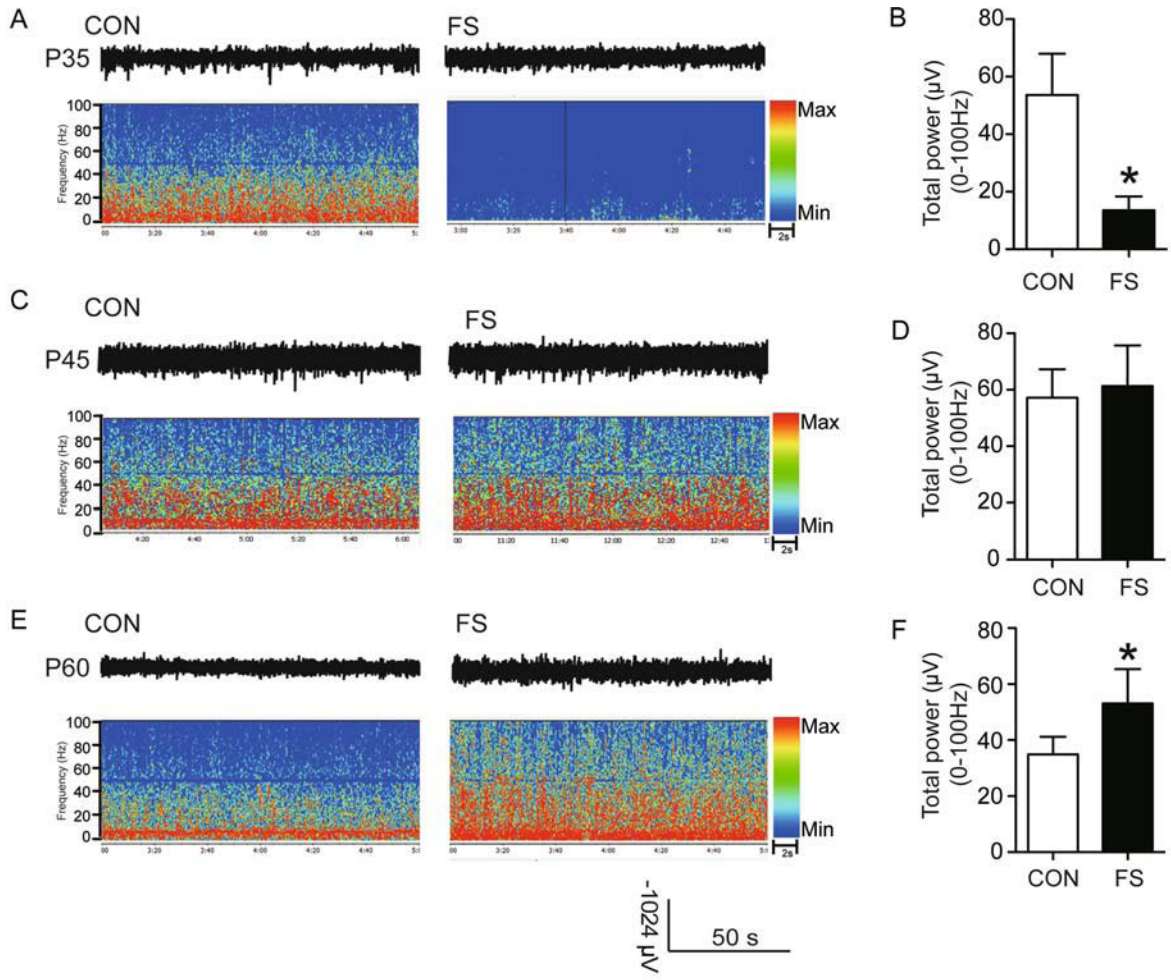

Fig. 3. Changes in the total power of hippocampal EEG after infantile FSs. (A, C, and E) Representative power spectra of hippocampal EEG recorded in control and FS rats for five consecutive days around P35, P45, and P60. (B, D, and F) Power in the total band of hippocampal EEG. *n* = 6 in control groups and *n* = 8 in FS groups. **P* <0.05 relative to the control group.

significant change in the power of δ, θ, and γ from P35 to P60 (Fig. 5A, B and E), but the power in α and β was slightly lower in adults (Fig. 5C and D). In the FS group, the power in the δ, θ, α, β, and γ bands all increased gradually from P35 to P60 (Fig. 5F–J). These data suggest the existence of a hypoactive period during epileptogenesis.

Neuroanatomical Matrix in Rats after FSs

Previous studies have reported that experimental FSs during adulthood lead to neuronal injury without detectable cell death in the hippocampus and amygdala[5]. Here, we examined the neuronal populations during the development of rats that experienced FSs. The neuronal counts did not differ between FS rats and the age-matched controls in CA1 (Supplementary Fig. 1B, D and F) and CA3 (Supplementary Fig. 1C, E and G). Then we quantified the neuronal cell body circumference occupied by GABAergic inhibitory presynaptic terminals in CA3 pyramidal neurons. In the P35 group, the coverage of neuronal surface by GAD^+ terminals was 39.7% in controls, and increased to 52.3% in FS rats (Fig. 6A). In the P45 group, the level of neuronal circumference occupied by GAD^+ terminals in the FS rats was similar to the age-matched controls (Fig. 6B, 43.8 ± 1.8% *versus* 41.2 ± 1.6%). In the P60 group, the coverage of neuronal surface by GAD^+ puncta was 41.9% in controls, and slightly reduced to 39.6% in P60 FS rats (Fig. 6C).

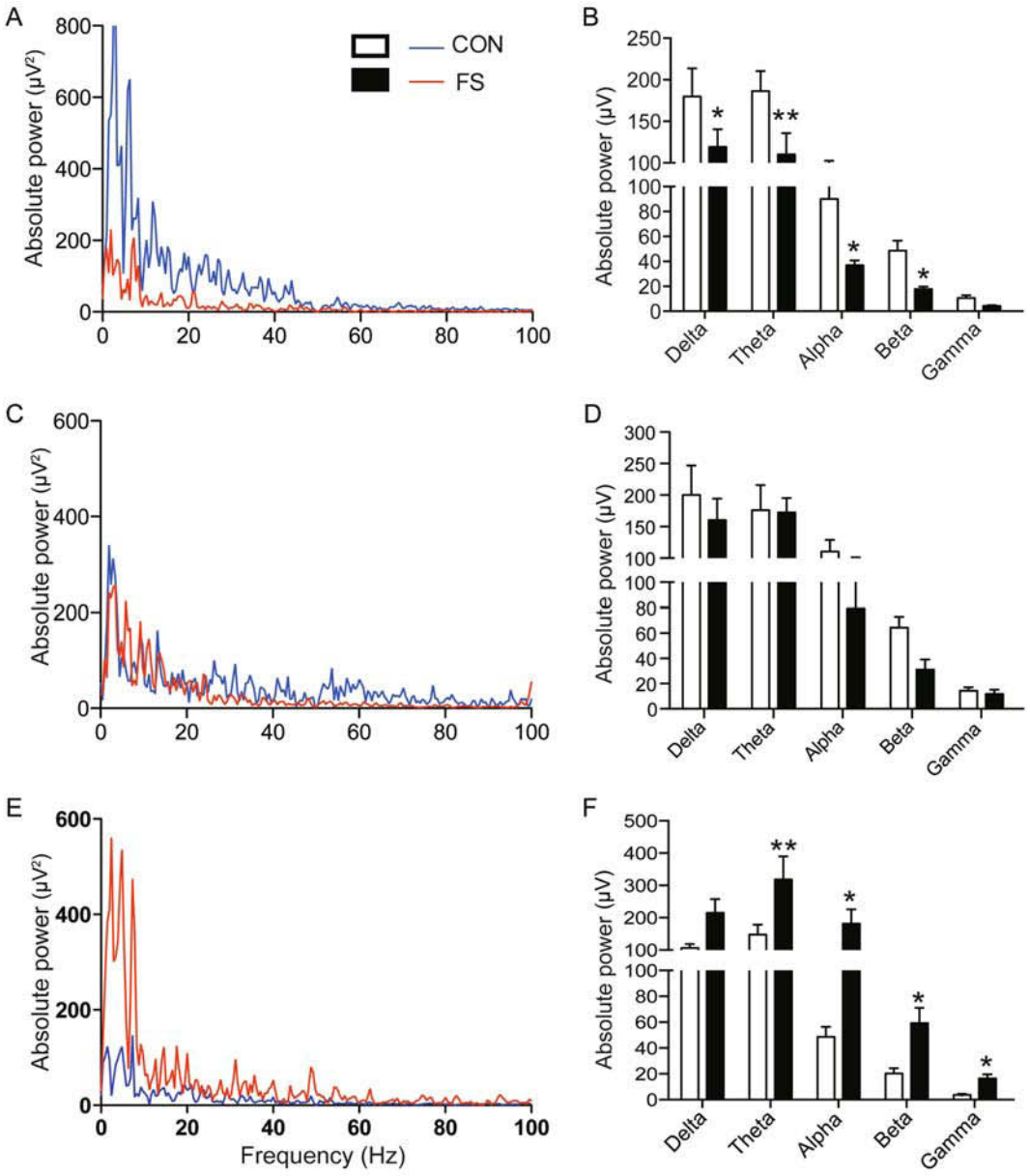

Fig. 4. Changes of the power spectra of hippocampal EEG after infantile FSs. (A, C, and E) Power spectra of the hippocampal EEG recorded in control (CON) and FS rats for five consecutive days around P35, P45, and P60, respectively. (B, D and F) Summary data from A, C, and E. *n* = 6 in control groups and *n* = 8 in FS groups (P* <0.05, ***P* <0.01 relative to the control group; two-way ANOVA with Bonferroni post-tests).**

DISCUSSION

In the present study, we revealed the following characteristics of epileptogenesis induced by FSs: (1) the seizure susceptibility showed dynamic changes from P35 to P60, but not a simple increase; and (2) before epileptic state or hyperactivity, there exists a hypoactive period in epileptogenesis, during which rats showed lower seizure susceptibility, decreased hippocampal EEG power in broad frequency bands, and increased coverage of the CA3 pyramidal neuronal surface by inhibitory synapses. Our data suggested that there is a hypoactive period in

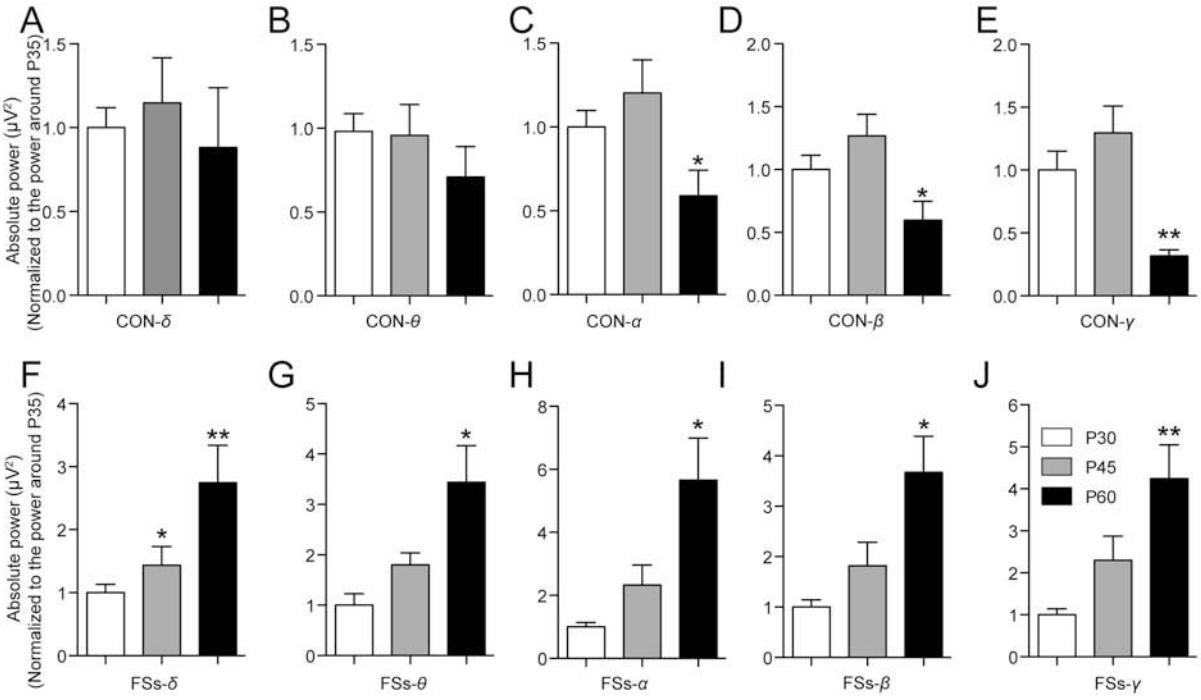

Fig. 5. Changes in the power spectra of hippocampal EEG in FS rats from P35 to P60. (A–E) Power spectra of δ, θ, α, β, and γ in the hippocampal EEG recorded in control (CON) rats from P35–P60, normalized to the power around P35. (F–J) Power spectra of δ, θ, α, β, and γ in the hippocampal EEG recorded from P35–P60 in rats that had experienced FSs, normalized to the power around P35. *n* = 8/group (P* <0.05, ***P* <0.01 relative to the normalized power spectra around P35; one-way ANOVA with Tukey's *post-hoc* test).**

the early phase of the latent period after FSs and a broad decrease of hippocampal rhythms may be a biomarker for this period, which may be important for early diagnosis of epileptogenesis induced by FSs.

Epileptogenesis is widely considered to be represented by a decrease in seizure threshold[21]. In accordance with this notion, we found that the seizure susceptibility increased from P30 to P60 in FS rats. Experimental and clinical studies support the idea that infantile FSs lead to an excitatory effect in adulthood, including enhanced long-term hippocampal excitability[5] and a reduced seizure threshold to excitatory input[4]. But few of these studies determined the seizure threshold during the early latent period. Here, we unexpectedly found that the susceptibility to MES-induced seizures in FS rats was lower than that in controls at P35, exhibiting lower seizure stages and a lower percentage of complete fore- and hindlimb extension. In addition, although the seizure susceptibility gradually increased after FSs, during the early phase it was significantly lower than that in controls. These results suggested that there is a hypoactive interval in the early phase of the latent period. Recent data indicate that administration of pro-convulsant drugs after traumatic brain injury or status epilepticus has anti-epileptogenic or disease-modifying effects[22, 23]. For example, a pro-convulsant cannabinoid CB1 receptor antagonist[24] prevents the limbic hyperexcitability caused in adults by developmental seizures during the immature period[23]. Our results may partly explain this phenomenon by showing the early-stage suppression and late-stage excitation after FSs and indicate that some pro-convulsant drugs might be used as anti-epilepsy drugs during the early phase of the latent period.

To characterize the early hypoactive period during epileptogenesis, we further recorded and analyzed the hippocampal EEG and found that the power was lower than that in controls at P35 but gradually increased through P35 to P60, consistent with the susceptibility change after FSs. This phenomenon may not be due to development, as the band power in controls was unchanged or decreased with increasing age. EEG recordings reveal

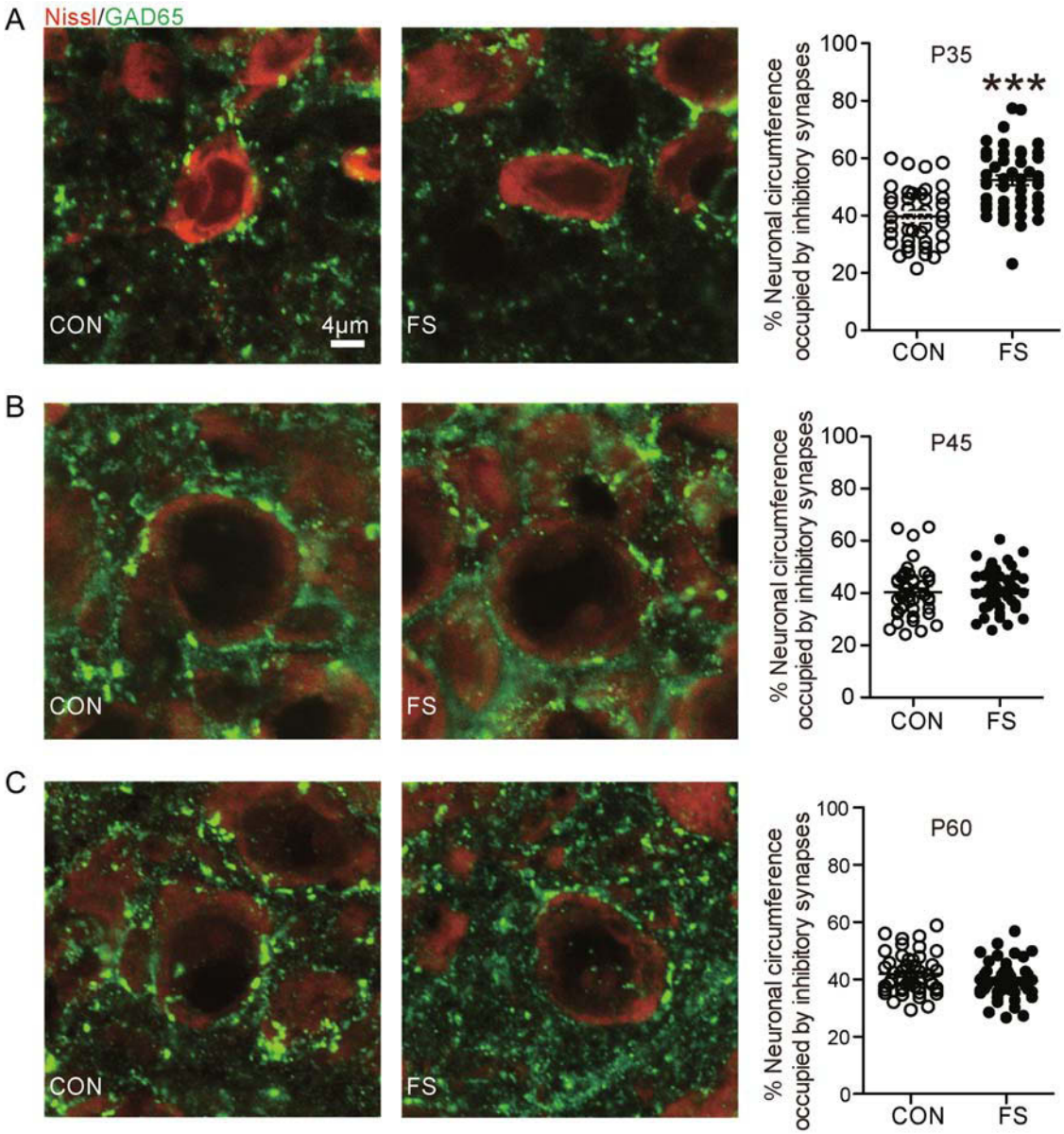

Fig. 6. Morphological changes in neuronal circumference occupied by inhibitory synapses. (A–C) Left: confocal micrographs of double labeling of neurons (red) and GAD$^+$ inhibitory synapses (green) in rats of different ages (scale bar, 4 μm). Right: statistical data (*n* = 5/group; ****P* <0.001 relative to age-matched control group; two-tailed Student's *t*-test).

functional connectivity patterns and dynamic changes in the epileptic brain, and can be used to predict upcoming seizures[24, 25]. So it is likely that the FSs disrupted the balance of excitation and inhibition during the latent period, which included a hypoactive interval in early phase. In accordance with this idea, previous studies have shown that glucose hypometabolism occurs in the limbic system in the early phase of the latent period after pilocarpine-induced status epilepticus[26, 27] and in the early stage of kindling acquisition[28], while the glucose metabolism recovers during the late phase of the latent period[26]. All this evidence suggests that the balance of inhibition and excitation is disrupted during the latent period, and inhibition predominates over excitation in the early phase. The broad decrease in hippocampal rhythms during this hypoactive period may be a candidate biomarker for the

early diagnosis of epileptogenesis.

During epileptogenesis after FSs, it is still unclear what changes in genes or molecules ultimately disrupt the balance of excitation and inhibition, reflected by the changes in EEG power[25]. Changes in hippocampal rhythms are associated with synaptic transmission, neuronal excitability, and neuronal loss. Since no neuronal loss occurred in adult rats[5] and during the latent period after FSs, the early broad decrease of hippocampal rhythms here may not be a result of neuronal loss. Instead, this may be due to (1) impaired synaptic development after FSs (e.g. GABAergically-induced ectopic granule cells in the dentate gyrus during development in FS rats[29]); and (2) a decrease in HCN (hyperpolarization-activated cyclic nucleotide-gated) channels, which are associated with hippocampal rhythms[31, 32], induced by FSs during epileptogenesis[30]. Here we found that the CA3 pyramidal neuronal cell body circumference occupied by inhibitory presynaptic terminals was significantly increased in FS rats at P35. Inhibitory input is closely associated with neuronal excitability. For instance, reduced inhibitory input makes neurons more excitable[20], and downregulation of GABA receptors to disrupt the inhibitory synapse leads to increased synchronization of neuronal firing[33]. Thus, the increased inhibitory synapses surrounding neurons found in our study may decrease the synchronization of neuronal firing during the early latent period, resulting in a lower seizure susceptibility and decreased hippocampal EEG power. A series of our previous reports has shown that the early brain network is crucial for epileptogenesis, and modulation of the early network can be a potential anti-epileptic strategy[16, 28, 34]. Therefore, modulation of the early hypoactive period may be effective in preventing epileptogenesis after early-life FSs.

In conclusion, the results of the present study show that there is a hypoactive but not hyperactive early phase in the latent period after FSs, displaying broad decreases of hippocampal rhythms. These characteristic EEG changes can be a useful biomarker for the early diagnosis of epileptogenesis induced by FSs.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s12264-014-1524-2.

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