

Temporal lobe epilepsy after experimental prolonged febrile seizures: prospective analysis

Céline Dubé,¹ Cristina Richichi,¹ Roland A. Bender,² Grace Chung,¹ Brian Litt³ and Tallie Z. Baram^{1,2}

Departments of ¹Anatomy/Neurobiology and ²Pediatrics, University of California, Irvine, CA and ³Department of Neurology and Bioengineering, Hospital of the University of Pennsylvania, Philadelphia, PA, USA

Correspondence to: Tallie Z. Baram, MD, PhD, Department of Anatomy and Neurobiology and Department of Pediatrics, University of California at Irvine, ZOT 4475, Irvine, CA 92697-4475, USA
E-mail: tallie@uci.edu

Experimental prolonged febrile seizures (FS) lead to structural and molecular changes that promote hippocampal hyperexcitability and reduce seizure threshold to further convulsants. However, whether these seizures provoke later-onset epilepsy, as has been suspected in humans, has remained unclear. Previously, intermittent EEGs with behavioural observations for motor seizures failed to demonstrate spontaneous seizures in adult rats subjected to experimental prolonged FS during infancy. Because limbic seizures may be behaviourally subtle, here we determined the presence of spontaneous limbic seizures using chronic video monitoring with concurrent hippocampal and cortical EEGs, in adult rats (starting around 3 months of age) that had sustained experimental FS on postnatal day 10. These subjects were compared with groups that had undergone hyperthermia but in whom seizures had been prevented (hyperthermic controls), as well as with normothermic controls. Only events that fulfilled both EEG and behavioural criteria, i.e. electro-clinical events, were considered spontaneous seizures. EEGs (over 400 recorded hours) were normal in all normothermic and hyperthermic control rats, and none of these animals developed spontaneous seizures. In contrast, prolonged early-life FS evoked spontaneous electro-clinical seizures in 6 out of 17 experimental rats (35.2%). These seizures consisted of sudden freezing (altered consciousness) and typical limbic automatisms that were coupled with polyspike/sharp-wave trains with increasing amplitude and slowing frequency on EEG. In addition, interictal epileptiform discharges were recorded in 15 (88.2%) of the experimental FS group and in none of the controls. The large majority of hippocampally-recorded seizures were heralded by diminished amplitude of cortical EEG, that commenced half a minute prior to the hippocampal ictus and persisted after seizure termination. This suggests a substantial perturbation of normal cortical neuronal activity by these limbic spontaneous seizures. In summary, prolonged experimental FS lead to later-onset limbic (temporal lobe) epilepsy in a significant proportion of rats, and to interictal epileptiform EEG abnormalities in most others, and thus represent a model that may be useful to study the relationship between FS and human temporal lobe epilepsy.

Keywords: prolonged febrile seizures; temporal lobe epilepsy; video-EEG; rat; prospective study

Abbreviations: FS = febrile seizures; TLE = temporal lobe epilepsy

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Introduction

Experimental prolonged febrile seizures (FS) can be evoked in infant rats at the age where hippocampal development is equivalent to that of human infants (Avishai-Eliner *et al.*, 2002). These seizures: (i) are limbic in semiology and involve the hippocampal formation (Dubé *et al.*, 2000); (ii) induce transient neuronal injury but no cell death (Toth *et al.*, 1998; Bender *et al.*, 2003a); (iii) lead to altered MRI T2-signal in

several limbic areas (Dubé *et al.*, 2004); (iv) cause profound and enduring alterations in the expression of several channel genes (Brewster *et al.*, 2002, 2005); and, (v) enhance hippocampal excitability long-term (Dubé *et al.*, 2000).

However, whether these prolonged (~20 min) experimental FS result in epilepsy, as has been suspected for the human condition (Annegers *et al.*, 1987; Hesdorffer and Hauser,

2002), has remained unclear. In the human, retrospective analyses have implicated early-life FS as a risk factor for the development of limbic, temporal lobe epilepsy (TLE; Cendes *et al.*, 1993; French *et al.*, 1993). However, whether early-life FS actually cause this TLE, or are simply indicative of other, perhaps genetically determined (Fernandez *et al.*, 1998; Wallace *et al.*, 2001) processes that eventually result in TLE, cannot be determined in correlative clinical studies. This question is particularly complex, because prospective studies in children with FS have not demonstrated the eventual development of TLE in the majority of those involved (Shinnar, 1998).

In the experimental model developed by the authors, adult animals that had endured prolonged FS early in life were originally monitored using intermittent, day-time EEG, without concurrent video recording or close behavioural observation. This approach did not demonstrate the development of spontaneous motor seizures after experimental prolonged FS. The lack of detection of epilepsy could be attributable either to a true absence of epileptogenesis after prolonged FS, or to missed spontaneous seizures. In view of the persistent hyperexcitability and the pro-epileptic molecular changes in hippocampi of rats that had sustained FS (*see above*), we tested the hypothesis that systematic video-EEG monitoring, particularly at night (Quigg *et al.*, 1998) may provide a more sensitive tool for investigating whether spontaneous seizures result from prolonged experimental FS.

Materials and methods

Animals and generation of experimental prolonged FS

Immature Sprague Dawley-derived rats were born and maintained in quiet facilities under controlled temperatures and light schedule with unlimited food and water. Cages were monitored every 12 h for the presence of pups and the date of birth was considered as day 0. On postnatal day (P) 2, litters were culled to 12 pups. When weaned (on P21), rats were housed 2–3 per cage. Experimental procedures were approved by Institutional Animal Care Committees and conformed to NIH guidelines.

Prolonged experimental FS were induced as described (Toth *et al.*, 1998; Dubé *et al.*, 2000). On P10, rat pups ($n = 17$) were placed in a 3 l jar and their core temperature raised using a

regulated stream of heated air to $\sim 41^\circ\text{C}$ (simulating high fever). Core temperatures (shown to correlate closely with brain temperatures; Dubé *et al.*, 2005a) were measured at baseline, seizure onset, and every 2 min during the seizures. This procedure leads to seizures in virtually all rats with a threshold at $\sim 40.8^\circ\text{C}$ (Dubé *et al.*, 2000). Hyperthermic temperatures ($40.2\text{--}41.5^\circ\text{C}$) were maintained for 30 min, and total seizure time was ~ 24 min. The behavioural seizures in this paradigm are stereotyped, consisting of arrest of heat-induced hyperkinesia followed by facial automatisms, often followed by body flexion. These behavioural events correlate with electrographic hippocampal seizures (Toth *et al.*, 1998; Dubé *et al.*, 2000; Brewster *et al.*, 2002; Bender *et al.*, 2003a). Hyperthermic controls ($n = 6$) were generated by subjecting littermates to the same duration of hyperthermia, but blocking the resulting seizures by treating the rats with the rapidly acting barbiturate pentobarbital (37 mg/kg, i.p) 15 min prior to the induction of hyperthermia. In one of these rats, brief behavioural seizures were observed during the first 2 min of hyperthermia, so it was excluded from analysis. An additional control group included littermates of the experimental group that were removed from the cage (to control for potential stress) and their core temperatures kept within the normal range for age (normothermic controls, $n = 8$).

Electrophysiological recording

EEG electrode implantation

Two months after prolonged FS (on postnatal day 81 ± 2), rats were implanted unilaterally with twisted wire bipolar electrodes with 0.5 mm vertical tip separation, into the dorsal hippocampus (using the coordinates AP = -3.7 , L = 2.7 , V = -3.7 mm with reference to bregma). Cortical electrodes (Plastics One, Roanoke, VA, USA) were positioned ipsilaterally over the frontoparietal cortex, and a ground electrode was placed in the cerebellum. The assembly was anchored with dental cement and four stainless steel screws. Hippocampal and cortical recordings were performed via long flexible cables (Plastics One, Roanoke, VA, USA) in freely moving, non-anaesthetized rats.

Long-term video-EEG monitoring

Starting one week after surgery, digital video-EEG recordings were carried out chronically, using 5 h sessions at night (from 11 p.m. to 4 a.m.; Quigg *et al.*, 1998). A minimum of five recordings was obtained per rat, on postnatal days 90, 105, 120, 135, 165 and 180 (*see Fig. 1*). Loss of the recording electrode assembly did not permit an equal number of sessions for each rat, and one animal was recorded over 11 sessions. In total, over 200 h of video-EEG were

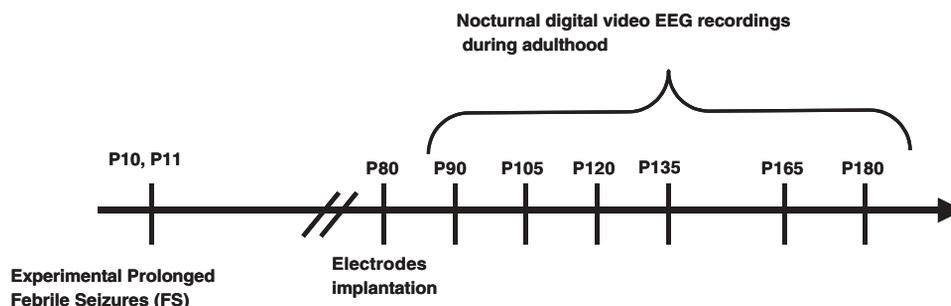


Fig 1. Study design.

recorded for each control group (normothermic and hyperthermic) and ~400 h were available for analysis for the prolonged FS group.

Electrophysiological data were recorded at a frequency band of 1–50 Hz, and sampled at 100 Hz/channel (4 channels) using Powerlab 8SP (AD Instruments, Grand Junction, CO, USA) equipped with Chart 4 for Windows. The EEG recordings were synchronized with video monitoring using a commercial video camera (camcorder, ZR40, Canon Inc, Japan).

Video and EEG review and definitions of electrographic and electro-clinical seizures

All analyses were carried out without knowledge of treatment. EEGs were first analysed visually, by browsing the digital records on a computer screen for seizures and for interictal events. Electrographic seizures were defined as follows (modified from Galvan *et al.*, 2000 and Nairismagi *et al.*, 2004): EEG parameters of electrographic seizures included the presence of polyspikes or sharp-waves (amplitude >2-fold background) that lasted >6 s. In addition, the progression of the amplitude and the frequencies of the discharges throughout a given seizure were analysed, because typical seizures are characterized by increasing amplitude and slowing frequency as the seizure progresses.

Interictal events were shorter than 5 s, and were not associated with behavioural change. They also lacked the typical progression of discharge frequency and amplitude (*see Results*). Video-taped behaviour concurrent with EEG changes was analysed. Typical behaviours associated with limbic seizures in humans and rodents, i.e. sudden cessation of activity, head bobbing or 'jerk', facial automatisms, prolonged immobility with staring, and alternating or bilateral clonus, were sought during these sequences. The final definition of a seizure required both electrographic and behavioural correlates, i.e. only electro-clinical events were considered seizures for the purpose of analysis.

For further analysis, data were exported from Chart 4 for Windows to Wave format and processed using MATLAB. The raw energy spectra of individual rat EEGs were calculated using fast Fourier transform. Each EEG recording was segmented into consecutive one second windows with 0.5 s overlap. Sequences containing severe movement artefacts were identified and excluded from analysis.

Histology and neuronal counts

At the conclusion of the video-EEG monitoring, rats were sacrificed, brains were rapidly removed and frozen on dry ice. For histological analysis, coronal serial sections (20 μm) were cut throughout the entire hippocampus using a cryostat. Neuronal counts were performed on every 25th section of a hippocampal series (yielding 9–10 sections from septal to caudal poles). These sections were stained with 1% cresyl violet and examined for electrode placement, then used for neuronal counts in hilus and CA1 of the dorsal hippocampus contralateral to the implantation site. Counts (under $\times 400$ magnification) were performed using stereological principles (Gundersen and Jensen, 1987), by an investigator unaware of the experimental group-status of the sections. Areas for analysis were defined as follows (Fig. 8A): the hilus was defined by its borders with the granule cell layer and by straight lines connecting the tips of the granule cell layer with the proximal end of CA3 pyramidal layer (Buckmaster and Dudek, 1997). In CA1, neuronal numbers were analysed in two, 250 μm wide vertical columns, the first positioned above the midpoint of the suprapyramidal blade of the granule

cell layer and the second at its tip. Neurons were included when their nucleus was clearly identifiable and was located >50% within the selected area. Abercrombie's correction (Abercrombie, 1946) was applied and neuronal densities (neurons/ mm^3) were then determined by dividing the extrapolated number of neurons (counts $\times 25$) by the volume calculated according to Cavalieri's principle (Gundersen and Jensen, 1987).

Statistical considerations

Prolonged FS experiencing animals were compared with age-matched control groups using ANOVA with *post hoc* tests or with Student's *t*-test, paired or not, as appropriate, using Prism software (GraphPad, San Diego, CA, USA). Values are reported as means \pm SEM.

Results

Experimental prolonged FS lead to behavioural and electrographic (electro-clinical) spontaneous seizures

EEGs (over 400 recorded hours) were normal in the normothermic and hyperthermic control rats. None of the control animals developed spontaneous seizures throughout the period of monitoring (>3 months). Early-life prolonged FS evoked electro-clinical (i.e. both behavioural and electrographic) spontaneous seizures in 6 of the 17 experimental rats (35.2% of total) and interictal events in 15 (88.2% of the group; Fig. 2).

Electrographic seizures recorded from unilateral bipolar hippocampal electrodes (Fig. 3A) consisted of polyspikes or sharp-wave trains with an amplitude that was at least twice the background and a duration of >6 s (consistent with the definitions of Galvan *et al.*, 2000, and Nairismagi *et al.*, 2004). As typical for seizures (Franaszczuk *et al.*, 1998; Bragin *et al.*, 1999) the amplitude of these events rose during the event (0.18 ± 0.01 mV at the onset of the 2nd second of the seizure and 0.41 ± 0.01 mV towards the seizure's end; $P < 0.0001$; paired *t*-test $n = 36$), and the frequency of discharges decreased (median frequency during the 2nd second of seizures was 9 Hz, whereas median frequency during the next-to-last second was 7 Hz. Means were 9.0 ± 0.2 Hz and 7.3 ± 0.2 Hz, respectively; $P < 0.0001$; paired *t*-test).

The behavioural correlates of these seizures consisted of sudden cessation of activity, accompanied by facial automatisms (stage 1; Racine, 1972). A head-jerk followed by gradual resumption of activity typically signaled the end of the seizure. The EEG changes recorded from unilateral hippocampal leads typically underestimated the duration of the behavioural seizures, and lasted 6–18 s (mean duration 7.8 ± 0.3 s, $n = 57$; Fig. 3B), consistent with involvement of other limbic regions in the generation of the behavioural phenomena (Ben-Ari *et al.*, 1981). In addition, these hippocampal EEG events were heralded and followed by far more widespread and longer changes in electrographic activity recorded from cortical leads.

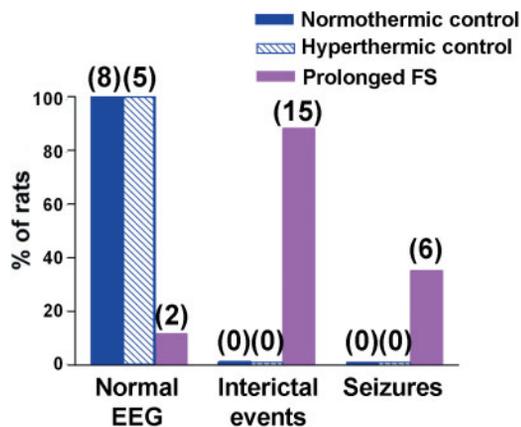


Fig. 2 Epilepsy after experimental prolonged febrile seizures (FS): Quantitative analysis of the number of rats developing epileptic EEG and behavioural changes. The prolonged FS group ($n = 17$) sustained hyperthermia for 30 min on postnatal day (P)10, that elicited seizures lasting 24.1 ± 0.1 min. Chronic video-EEGs were recorded during P90–180 in this group and in normothermic and hyperthermic control rats (see Materials and methods for definitions). In both control groups, EEGs recorded over more than 400 h did not contain any epileptiform discharges. None of the controls developed spontaneous seizures. The FS evoked interictal events in 88.2% of rats, and spontaneous seizures, with both behavioural and electrographic manifestation, seizures in six (35.2%) rats.

In the cortical EEGs, the amplitude decreased substantially starting about half a minute before the hippocampal onset of the seizures (23.9 ± 5.2 s; $n = 44$). This low amplitude cortical activity endured during the electrographic hippocampal seizures and persisted for 15.2 ± 3.1 s after the apparent end of the paroxysmal hippocampal activity (Fig. 4A). Thus, although the electrographic events recorded from unilateral hippocampal electrodes were short, the overall neuronal disturbance associated with these seizures was substantially longer: reduced amplitude of cortical EEG lasted on average 49 ± 6.4 s, and in some cases up to 4 min (Fig. 4B).

Postictal depression was apparent in hippocampal traces after termination of the seizures (Fig. 5A). This pattern was evident in 50% of rats, and mean duration of this depression was 3.2 ± 0.4 s (Fig. 5B). Note that cortical postictal depression was significantly longer, lasting 15.2 ± 3.1 s ($P < 0.05$, Fig. 5B).

Spontaneous seizures were already present in the first video-EEG session in 50% of epileptic rats, permitting initial assessment of their progression with time over the 3 month recording period. When feasible, this analysis did not reveal progressive increase of seizure frequency or duration.

Experimental prolonged FS alter hippocampal neuronal function, manifest by interictal epileptiform discharges

Prolonged FS caused interictal events in 15 of 17 rats (88.2%) that were detected primarily during periods of rest.

These events were not found in normothermic or hyperthermic control groups, excluding the possibility that they were variants of normal oscillatory activity within hippocampus (Wiest and Nicolelis, 2003; Kelly, 2004). Interictal events were defined using several criteria that clearly distinguished them from seizures: First, when those lasting 4–5 s were analysed, there was no increase of discharge amplitude between the first and last seconds: 0.20 ± 0.01 mV and 0.21 ± 0.01 mV, respectively, $n = 26$. Frequency of discharges during these events also did not change (8.35 ± 0.32 Hz during the first second versus 8.12 ± 0.31 Hz during the last, $n = 26$). In addition, the raw spectra of seizures and of interictal events differed: seizures were characterized by a major sharp peak (amplitude >8-fold of basal) of 7 Hz activity, with a minor (~2-fold amplitude increase) additional spike at 23 Hz. In contrast, a similar analysis of the interictal events found a broad ‘hump’ of frequencies at 5–9 Hz, as well as several smaller peaks at ~17–18 Hz, 25 and 26 Hz (data not shown). Both types of events also had components in the gamma, 36–46 Hz range. Thus, the contour of the interictal events was typical of limbic epileptiform interictal discharges, consisting of polyspikes and/or sharp-waves (50–200 ms) in 87% of rats (Fig. 6A). Interestingly, quantitative analysis of the duration of interictal events in epileptic rats (Fig. 6B) revealed a temporal distribution that tended to be consistent for each rat, and different among individual animals (Fig. 6C, e.g. duration of events of rat no. 3 versus nos. 1, 4 or 5, $P < 0.05$).

Hippocampal electrographic activity of rats that develop epilepsy after experimental prolonged FS is persistently altered

We queried whether fundamental neuronal oscillatory activity, outside of periods of frank seizures, was altered by the early-life FS. Therefore, we analysed the raw EEG energy spectra in individual rats that developed spontaneous seizures ($n = 6$) compared with those manifesting interictal events only ($n = 9$), and normothermic controls ($n = 8$). Remarkably, energy spectra of the three groups differed, with a striking reduction of low-frequency (1–10 Hz) energy found in the frankly epileptic rats (Fig. 7). This reduction did not occur in FS-experiencing rats that did not develop spontaneous seizures: in the latter group, energy spectra resembled those of controls.

Neuroanatomical matrix of epileptogenesis in rats after experimental prolonged FS

We have shown previously that experimental prolonged FS lead to transient neuronal injury, but no detectable cell death in hippocampus and amygdala, as well as in limbic cortices (Toth *et al.*, 1998; Bender *et al.*, 2003a). However, in these original studies, we did not distinguish between animals that became epileptic and those that did not. Thus, the question

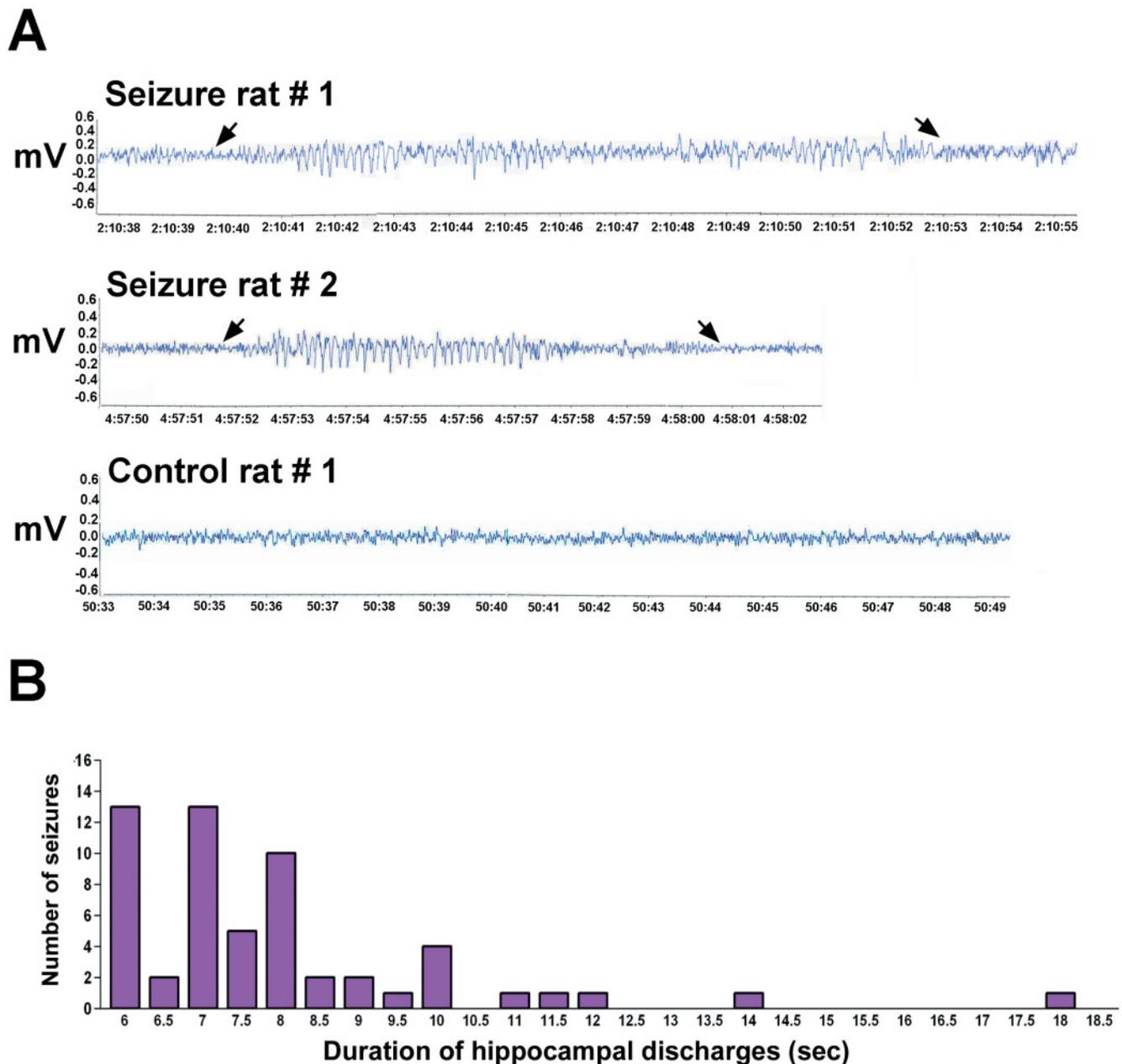


Fig. 3 Typical spontaneous electrographic seizures recorded from hippocampal electrodes in adult rats that had sustained prolonged febrile seizures (FS) early in life. Seizures were defined as events meeting both EEG and behavioural criteria: electrographic parameters included presence of polyspikes or sharp-wave trains lasting over 6 s (see Materials and methods). **(A)** Hippocampal EEGs. Arrow points to onset and end of epileptiform discharges. The typical behaviour associated with these events was sudden cessation of activity accompanied by facial automatisms. Typically, behaviour changes slightly preceded the onset of hippocampal seizures, and lasted longer. Hippocampal EEG from a normothermic control rat shows low amplitude baseline trace. **(B)** Histogram of the duration of hippocampal EEG-recorded spontaneous seizures in adult rats that had endured prolonged FS early in life.

of whether epileptogenesis after prolonged FS is accompanied by (and may require) neuronal loss has not been fully resolved.

This question was addressed in the current study by quantifying neuronal populations specifically in those rats that had developed epilepsy after FS. We chose to examine populations known to be most vulnerable to

seizures in animal models of limbic epilepsy (i.e. hilar neurons; Sloviter, 1994), as well as those most affected in human TLE after an early-life precipitating event including prolonged FS (i.e. neurons in Sommer's sector = CA1; Armstrong, 1993). We did not examine neuronal counts in the entorhinal cortex. As shown in Fig. 8, neuronal densities did not differ between the epileptic and the

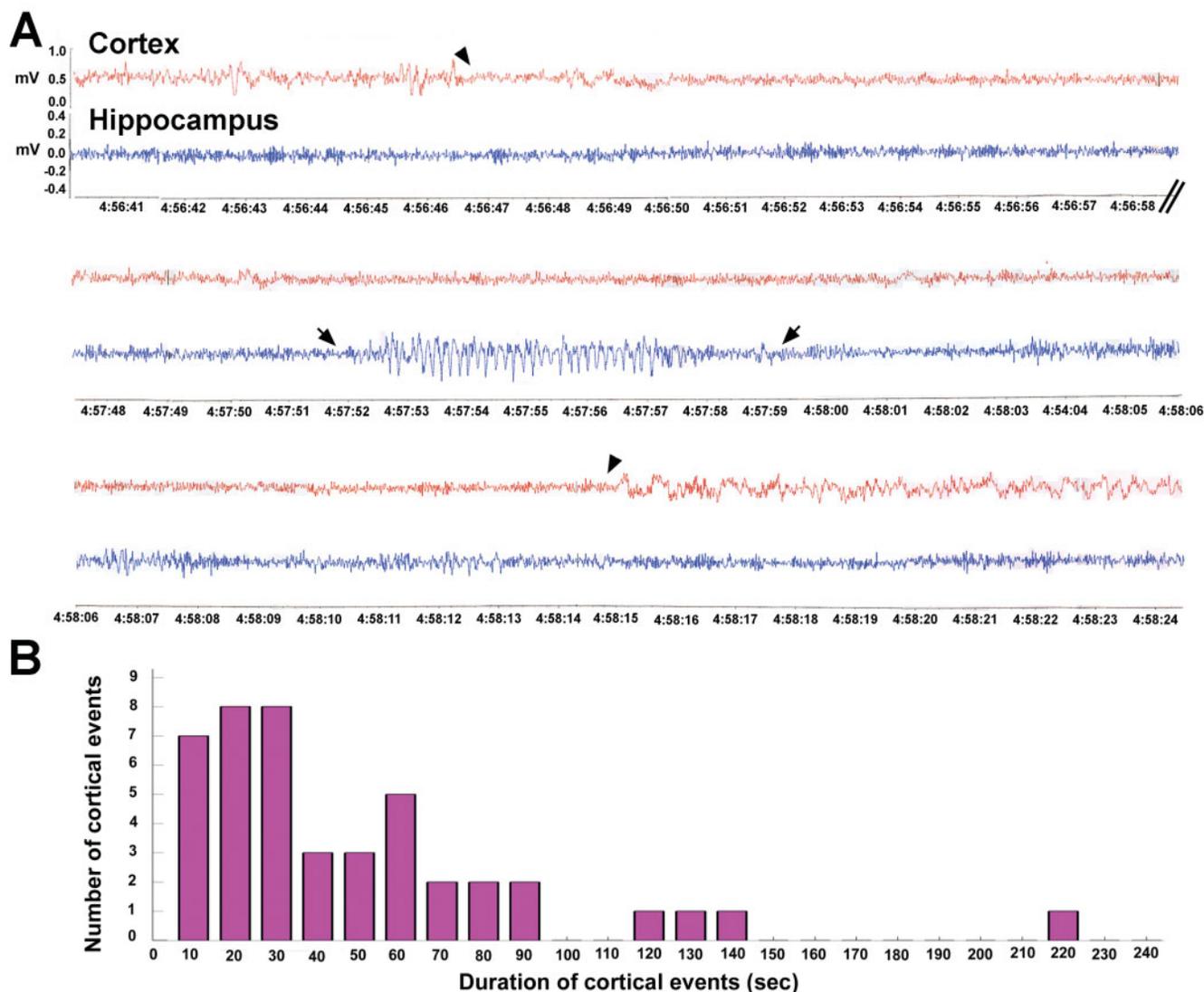


Fig. 4 Perturbation of cortical activity during spontaneous seizures evoked by experimental febrile seizures (FS) precedes and outlasts hippocampal discharges. **(A)** Example of reduced cortical EEG amplitude (indicated by the arrowheads) occurring before the onset of a hippocampally-evident spontaneous seizure and persisting during and beyond it, for a total duration of 92 s (arrows denote the onset and the end of hippocampal epileptiform discharges). **(B)** Histogram of the duration of these cortical perturbations during seizures in rats rendered epileptic by prolonged FS early in life. Note that reduced amplitude of cortical EEG lasts on average 49 ± 6.4 s, and in some cases up to 4 min.

normothermic control group in both the hilus (Fig. 8B; $P = 0.16$) and CA1 (Fig. 8C; $P = 0.93$). In addition, no changes of hilar or hippocampal volume were observed that might confound the cell counts (Fig. 8D).

Discussion

The major findings of these studies are (i) Prolonged experimental FS induce recurrent, spontaneous, behavioural and electrographic (electro-clinical) seizures later in life in 35% of rats (and never in controls). (ii) The ictal events are brief, but perturb normal cortical neuronal activity for significantly longer periods, evident by reduced cortical EEG

amplitude. (iii) Fundamental EEG activity of rats rendered epileptic after FS is modified, constituting a potential marker for epileptogenesis after prolonged FS in the human. Taken together, the data indicate that experimental prolonged FS may provoke limbic epilepsy, and may therefore represent a useful model for studying the relationship between FS and human TLE.

Early-life prolonged 'FS' lead to the emergence of spontaneous seizures that involve the hippocampus

Using an established model of early-life prolonged FS (Toth *et al.*, 1998; Brewster *et al.*, 2002; Bender *et al.*, 2003a; Dubé

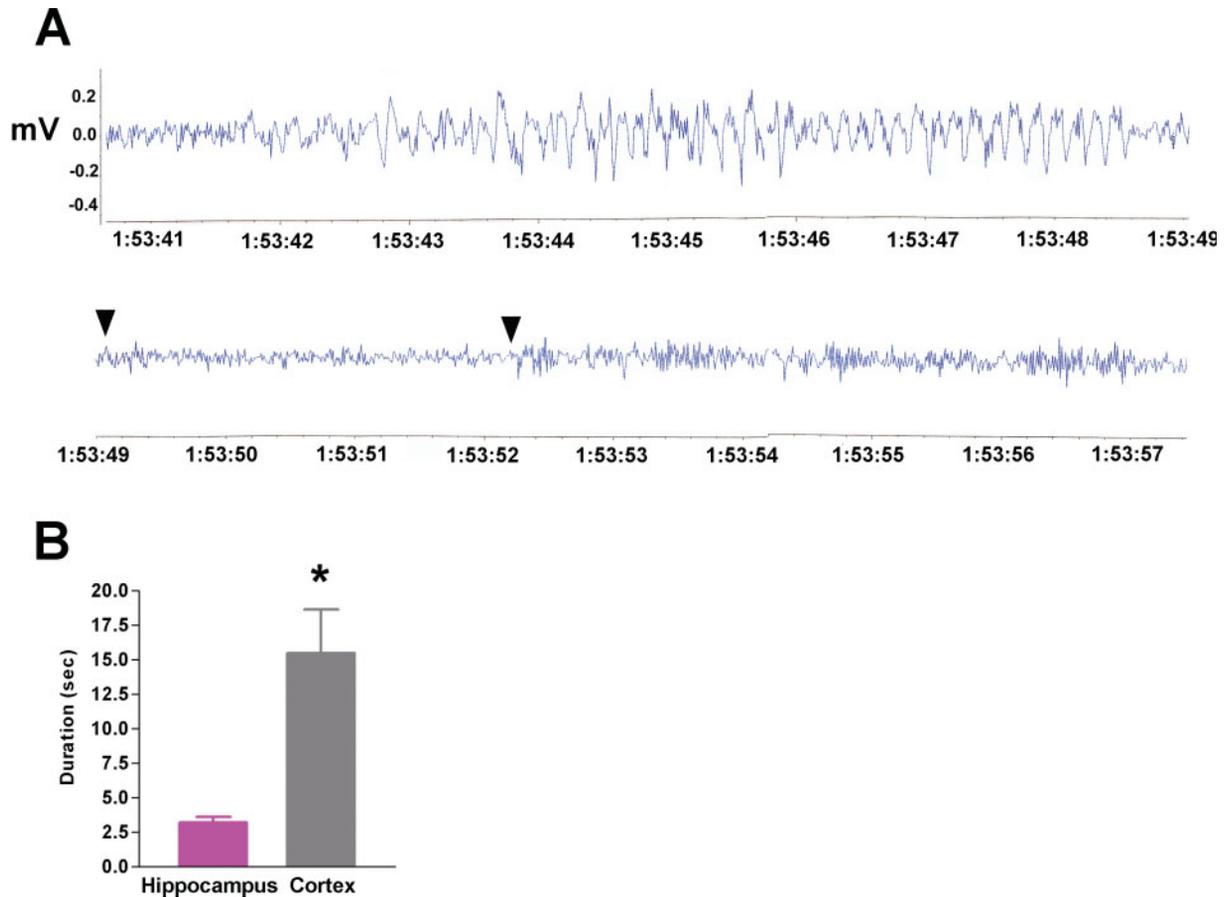


Fig. 5 Postictal depression in hippocampal EEG follows spontaneous seizures evoked by early-life febrile seizures. **(A)** An example of postictal depression (arrowheads denote the onset and end of the depression) occurring immediately following the seizure. **(B)** Comparison of the mean durations of hippocampal and cortical postictal depressions, showing that hippocampal postictal depression averaged 3.2 ± 0.4 s, whereas cortical EEG postictal depression lasted 15.2 ± 3.1 s. * $P < 0.05$.

et al., 2004), the current study demonstrates that these convulsions lead to spontaneous hippocampal seizures, i.e. they generate limbic epilepsy. The seizures recorded to date have been brief and were defined as ictal when several conditions coincided: epileptiform hippocampal EEG discharges exceeded 6 s, they were typical of seizure progression, with increasing amplitude and slowing frequency, and were associated with abruptly modified behaviour. The behaviours, in turn, were in accord with those found in human (and experimental) limbic seizures when the seizures are confined to the limbic circuit (Margerison and Corsellis, 1966; Goddard *et al.*, 1969; Racine 1972; Theodore *et al.*, 1983; Williamson *et al.*, 1987). This conservative approach that defined only electro-clinical events involving both behaviour and abnormal neuronal activity as seizures, may have underestimated the number of seizures, because epileptiform EEG activity without clearly evident behavioural changes was observed on occasion.

The events recorded from unilateral hippocampal electrodes in this study were short (6–18 s). In many cases, behavioural changes, typically freezing (Racine-stage 0), preceded the onset of hippocampal discharges by ~ 1 –2 s, and return

to normal behaviour was delayed by 2–3 s after termination of hippocampal EEG seizures. This is to be expected if the hippocampus, where the recording electrode was placed, was not the site of origin of the seizures. Indeed, the origin of seizures within the limbic circuit is often outside of the hippocampus (Ben-Ari *et al.*, 1981), not uncommonly in the amygdaloid complex. Therefore, given that we had electrodes only in a single dorsal hippocampus, the apparent dissociation between the onset of the clinical and the EEG onset of the seizures suggests, or is at least consistent with, a seizure origin elsewhere in the limbic circuit. Thus, our results for the duration of these seizures are probably an underestimate.

Whereas most human limbic seizures are longer than the seizure duration recorded here, the minimal duration required to consider epileptic events as seizures is not well defined. In humans, absence seizures are often defined as lasting 3 seconds or longer, and thus leading to apparent alteration of consciousness (International League Against Epilepsy, 1981). In animal models, many definitions exist (Bragin *et al.*, 1999; Galvan *et al.*, 2000; Raol *et al.*, 2003; Nairismagi *et al.*, 2004). In addition, seizures have even been

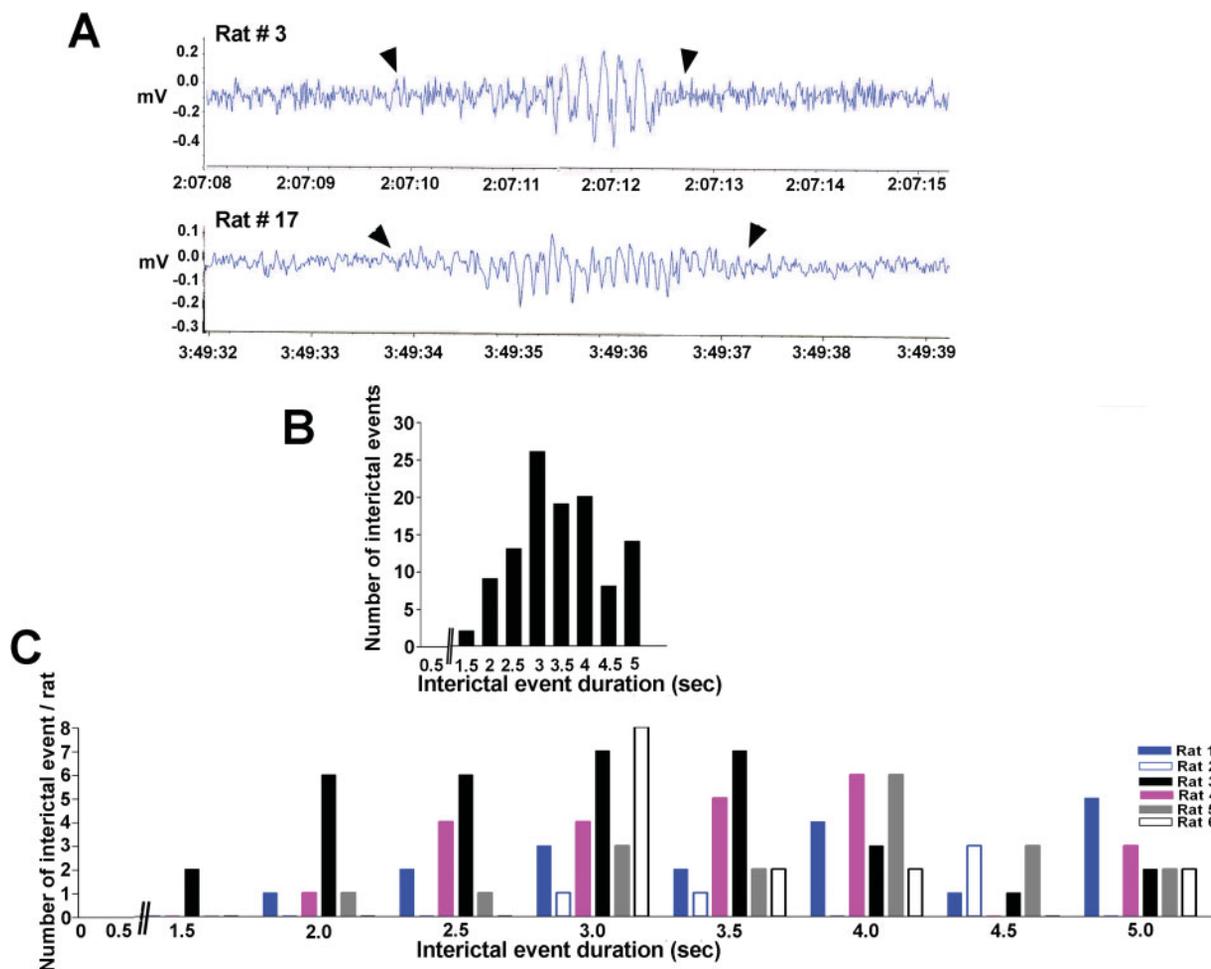


Fig. 6 Interictal epileptiform activity recorded from hippocampal electrodes in adult rats that had endured prolonged febrile seizures early in life. **(A)** Examples of interictal epileptiform activity (arrowheads), defined as polyspikes or sharp-waves shorter than 5 s, and without altered behaviour. **(B)** Histogram of the duration of the interictal discharges in the six epileptic rats. The distribution indicates that the majority of these events lasted between 2.5 and 4 s. **(C)** Histogram of the duration of the interictal epileptiform discharges analysed by rat. The distribution of the duration of these events seems to differ among individual animals. e.g. event durations in rat no. 3 were significantly lower than those in rats nos. 1, 4 or 5, $P < 0.05$.

recognized, with no lower limit of duration, also in *in vitro* systems (Khalilov *et al.*, 2003). Common operational definitions of temporal lobe (limbic) seizures in humans rely on epileptiform EEG activity that suffices to alter behaviour (and/or consciousness). In view of these non-uniform approaches, seizures in the current study have been defined operationally, as ictal events that abruptly influence behaviour. In addition, both parameters, the EEG and the behaviour, have further been subjected to biological-relevance constraints: The EEG activity signifying a seizure must be epileptiform, as conventionally defined (amplitude larger than twice the background, spikes/polyspike contour, progression of both amplitude and frequency). In analogy, the behaviour should be consonant with that exhibited by humans and animals (e.g. early phases of kindling) during verified, well accepted seizures that are confined to the limbic circuit (Goddard *et al.*, 1969; Racine 1972; McIntyre and Kelly, 1993). The events described here

meet these conservative criteria, and are thus considered seizures.

Short spontaneous hippocampal seizures after experimental FS involve longer and wider disturbances in cortical oscillatory activity

The ictal events recorded from hippocampal electrodes were associated with longer and more wide-ranging electrographic changes typical of limbic seizures in humans and animal models: starting about half a minute prior to the onset of hippocampal epileptiform discharges, cortical EEG amplitude suddenly declined. This 'depression' persisted during the hippocampal seizures and into the hippocampal postictal period, lasting up to 4 min. In addition, slowing of frontoparietal EEG activity during the seizures occurred in a quarter of the events, reminiscent of ictal slowing in frontal

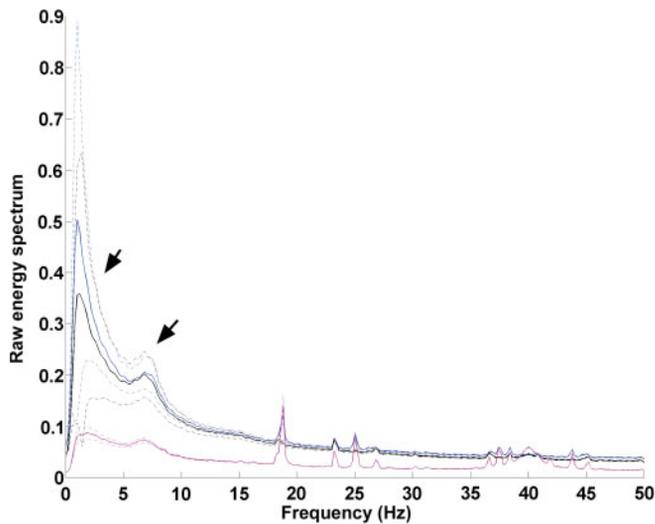


Fig. 7 Raw energy spectra of individual nocturnal hippocampal EEGs from rats with febrile seizures (FS)-evoked spontaneous seizures (pink; $n = 6$) compared with FS-experiencing rats with epileptiform interictal discharges only (black; $n = 9$) and to normothermic control rats (blue; $n = 8$). A significant reduction of low-frequency energy (arrows) is apparent in rats with spontaneous seizures (epileptic), suggesting that these seizures may perturb global neuronal network activity. For each group, the solid line represents the mean of the raw energy; dashed line denotes the standard errors.

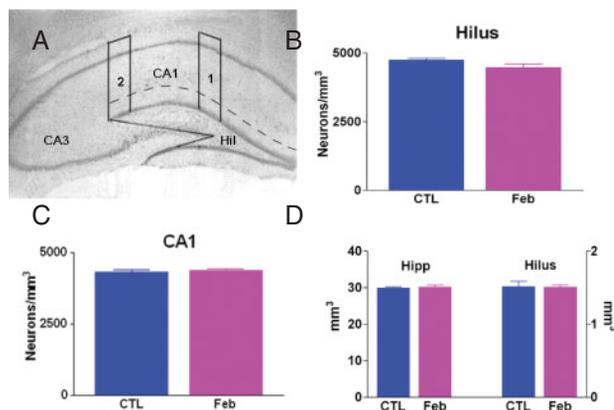


Fig. 8 Neuronal density analyses in hippocampal regions most affected in temporal lobe epilepsy do not indicate cell loss in epileptic rats. Analyses were performed on sections subjected to Nissl stain. This stain permits distinction between neurons (large nuclei, ample cytoplasm with abundant ‘Nissl material’ (ribosomes) and astrocytes (smaller nuclei, cant pale cytoplasm). (A) Illustration of areas selected for data sampling in hilus and CA1 (see Materials and methods). Neuronal densities in hilus (B) and CA1 (C) of rats that had become epileptic after early-life prolonged febrile seizures (pink bars) did not differ from densities in normothermic controls (blue bars). (D) Dorsal hippocampal (left y-axis) and hilar volume (right y-axis) were the same in epileptic and control rats, excluding volume changes as a potential confounder of these analyses.

and ipsilateral parietal EEGs of human temporal lobe seizures (Lieb *et al.*, 1991; Blumenfeld *et al.*, 2004). This slowing is considered to derive from surround inhibition of the structures outside the area of seizure onset (Engel *et al.*,

1982; VanLandingham and Lothman, 1991; Schwartz and Bonhoeffer, 2001). In addition, postictal depression, i.e. reduced amplitude of EEG discharges, was evident in hippocampal EEGs in 50% of animals and a third of the ictal events, as found in human postictal EEGs (Ajmone-Marsan and Ralston, 1957; Kaibara and Blume, 1988; Franaszczuk *et al.*, 1998; Olejniczak *et al.*, 2001). These findings suggest that these spontaneous seizures evoked by early-life experimental FS perturbed normal neuronal oscillatory activity in cortical and limbic circuits for substantially longer periods than their apparent duration.

In addition, power spectral analysis of the EEGs of rats that developed spontaneous seizures suggested remarkable alterations in the overall rhythm spectrum of neuronal activity. Thus, EEGs from epileptic rats were characterized by a significant reduction of energy in the low frequencies (1–10 Hz). This was unique to this group, and distinguished it from the group with interictal events alone, where EEG spectra resembled those in controls. Whereas the precise relationship of the loss of the low-frequency oscillation (typically associated with sleep) and the presence of spontaneous seizures is not clear, it is tempting to speculate that the seizures may disrupt these normal brain activities, potentially via alteration of sleep architecture (Besset, 1982; Bazil *et al.*, 2000).

Presence of epileptiform ‘interictal’ activity in most FS-experiencing rats suggests that experimental prolonged FS modify hippocampal circuits

Prolonged FS led to interictal events in 88.2% of the rats (and to frank seizures in 40% of this group). These data suggest that the seizures influenced neuronal inter-communication in the limbic circuit in the majority of animals. Whereas the mechanisms underlying these FS-evoked changes are not well understood, previous studies have excluded potential candidate mechanisms and implicated others. Thus, experimental prolonged FS did not result in death of any of the neuronal populations that are vulnerable to prolonged seizures in adult (Ben-Ari, 1985; Sutula *et al.*, 1988; Sloviter *et al.*, 1994; Pitkänen *et al.*, 2002) and juvenile rats (Sankar *et al.*, 1998; Kubova *et al.*, 2001), or are injured or lost in human TLE (Falconer *et al.*, 1964; Armstrong, 1993; Mathern *et al.*, 2002). Interestingly, transient cytoskeletal changes in several of these populations, including hippocampal CA1 and CA3 pyramidal cells and some hilar neurons were found after experimental FS (Toth *et al.*, 1998). In addition, MRI studies have demonstrated acute T2 signal changes after these seizures in limbic structures, partially overlapping those involved in the cytoskeletal studies. These occurred in a majority of FS-rats, and were not a result of neuronal death (Dubé *et al.*, 2004, Fig. 4).

Whereas the studies described above analysed all animals after experimental FS, here we counted vulnerable neuronal populations specifically in rats that became epileptic after

experimental FS, compared with normal controls. Again, we found little evidence of cell loss in the epileptic animals, although, in the absence of rigorous stereological methodology in this study, loss of a minimal number of neurons cannot be absolutely excluded. This lack of significant neuronal loss suggests that other mechanisms, not requiring cell loss, become operative after experimental FS to provoke epilepsy in these animals (Baram *et al.*, 2002). These probably include substantial functional changes in the properties of hippocampal neurons. For example, early and sustained increase in GABAergic inhibition was found in animals that had experienced experimental FS (Chen *et al.*, 1999). Importantly, neuronal excitability was augmented (Dubé *et al.*, 2000) at least in part by increased hyperpolarization-activated current, I_h , promoting rebound depolarization in response to hyperpolarizing input (Chen *et al.*, 2001; Santoro and Baram, 2003). The basis for this increase of I_h , carried by the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, involved seizure-evoked alteration of expression of HCN channel isoforms, with a consequent change of the current they carry. Enhanced expression of the cyclic-AMP (cAMP) sensitive slow-deactivating HCN2 isoform, reduced expression of the fast-deactivating, relatively cAMP insensitive HCN1, and formation of heteromeric channels composed of these two isoforms probably contribute to augmented hyperpolarization-dependent rebound depolarization in hippocampal neurons (Brewster *et al.*, 2002, 2005; Santoro and Baram, 2003).

Relevance of the study to human epilepsy, limitations and future studies

This is the first study that directly addresses the causal relationship of long FS and the development of TLE. In the animal model, where predisposing factors, both genetic (e.g. Fernandez *et al.*, 1998; Wallace *et al.*, 2001) and acquired, have largely been excluded, the answer is in the affirmative. However, many caveats and further questions remain. First, spontaneous seizures developed only in a subgroup of rats experiencing prolonged FS (a minimum 35%, although this may be an underestimate, as discussed above), and the reasons are not clear. Whereas occult genetic factors may have not been fully excluded and might contribute to such variability, model-related parameters are more likely to account for the development of epilepsy only in a subset of animals. For example, sub-clinical, longer-duration seizures may have persisted beyond the original hyperthermia-evoked 'FS' in some animals, promoting the development of epilepsy. Alternatively, subtle undetected seizures may have recurred during the first few hours after the inciting hyperthermia-induced FS. These questions will be addressed in future studies. Larger group sizes will also better estimate the precise proportion of animals that develop TLE, and, to eliminate the possibility that electro-clinical seizures were missed because the EEG-epileptiform discharges originated from the hemisphere contralateral to that of the recording electrodes, bilateral

hippocampal electrodes will be employed, as well as extra-hippocampal (such as amygdala) leads (Gloor, 1991). Finally, whereas the current recordings were prolonged, they were mainly carried out at night and not continuously for weeks and months. The availability of telemetric devices should facilitate future continuous-recording studies. These studies will also permit us to determine the exact time when the 'pro-epileptic' EEG features of the seizure-experiencing rats arise.

In summary, this is the first direct study examining the consequences of the most common seizures of childhood on the development of TLE. In this study, prolonged experimental FS provoked limbic (temporal lobe) epilepsy. Therefore, this seizure model provides a powerful tool for further addressing important questions in human epilepsy. These include the mechanisms by which these seizures promote epileptogenesis (Bender *et al.*, 2003*b*; Dubé *et al.*, 2005*b*), the presence of markers for this process (Roch *et al.*, 2002; Dubé *et al.*, 2004; Nairismagi *et al.*, 2004), and the potential for interventions in the epileptogenic process (White, 2002).

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