nature neuroscience

In vitro formation of a secondary epileptogenic mirror focus by interhippocampal propagation of seizures

Ilgam Khalilov[1], Gregory L Holmes[2] & Yehezkel Ben-Ari[1]

We have determined whether seizures generate an epileptogenic focus in distal structures using an *in vitro* preparation composed of three independent chambers that accommodate two intact hippocampi and their connecting commissures. This enabled us to apply a convulsive agent to one hippocampus, allow the propagation of a given number of seizures to the other side and block the connections reversibly by applying tetrodotoxin (TTX) to the commissural chamber. The propagation of seizures from the kainate-treated side to the naive side transformed the latter into an independent epileptogenic focus that was capable of generating spontaneous and evoked seizures. The induction mechanism required activation of NMDA receptors and the epileptogenic transformation was associated with long-term alterations in GABAergic synapses, which became excitatory because of a shift in the chloride reversal potential, E_{Cl}. These data indicate that the excitatory actions of GABA may be a fundamental property of epileptogenic structures.

A fundamental concept in epilepsy is that the propagation of paroxysmal activity is necessary and sufficient to transform a naive structure into one that is capable of generating spontaneous and evoked seizures. This transformation is thought to underlie the formation of an epileptogenic focus that will entrain the generation of seizures in interconnected structures. In keeping with this, experimental studies *in vivo* indicate that seizures may produce long-term consequences in neuronal structures that extend beyond the epileptic focus[1,2]. Normal brain tissue may develop an enduring functional alteration with enhanced epileptogenic properties as a consequence of being exposed to repeated seizures that originate outside that region. This phenomenon, termed secondary epileptogenesis, has many implications for both clinical and basic research. Patients with active epileptogenic regions may develop secondary epileptogenesis at a site distant from the original focus, a factor that may reduce the likelihood of successful surgical treatment of the epilepsy[3]. Other studies, however, cannot be reconciled with this, as most adult patients, despite years of epilepsy, do not develop secondary epileptogenesis and can undergo successful surgical treatment[4]. Nevertheless, if repeated seizures do lead to permanent adverse consequences in distal structures, it is important to determine the mechanisms involved to understand better the abnormal functioning of the epileptic brain.

The problem of secondary focus may be particularly important in the developing brain. Seizures are more common in the immature brain than in the adult brain[2,5–8] and lead to long-term neurological disorders without necessarily causing substantial neuronal cell loss[2,7,9]. Therefore, the adverse effects of seizures on the developing brain are probably mediated by alterations in activity-dependent processes that are modulated by neuronal activity. Processes known to be modulated in this way include cell division, neuronal growth and migration, receptor clustering and synapse formation[10–13]. Thus, it is important to determine how recurrent seizures can permanently alter developmental programs. In addition, dual pathology is most commonly seen in children with developmental disorders such as neuronal migration disorders[3,14]. Using clinical studies, it is difficult to determine whether this occurs as a result of the development of a secondary epileptic focus in normal brain or because of subtle pathological lesions at sites distant from the primary pathology. Animal studies are therefore essential in determining whether epileptiform activity can elicit an independent epileptic focus at sites distant from the original seizure focus. The inherent limitations of *in vivo* studies, which include the diffusion of convulsive agents, the complex local and distal effects of electrical stimulations and the effects of back-propagating seizures, highlight the need for an *in vitro* preparation to determine whether recurrent seizures generate a secondary focus.

In the present study, we have provided direct evidence that repetitive seizures lead to the formation of a secondary epileptogenic focus in the immature brain. We have used an *in vitro* preparation composed of two interconnected hippocampi and their commissural fibers, which are dissected intact. The hippocampi and commissural fibers are placed in three independent chambers that are designed to allow a selective perfusion of each compartment[15,16]. It is thus possible to apply a convulsive agent to one hippocampus, follow the propagation of seizures to the naive side and reversibly block the connections by selectively applying the sodium channel blocker TTX to the commissural chamber. We report that the propagation of ictal activity from one hemisphere to the other generates a quasi-permanent change of the naive circuit, which then generates spontaneous and evoked seizures when isolated from the stimulated side. NMDA receptors must be activated to generate a secondary focus, suggesting a persistent increase in synaptic efficacy. GABA exerts an excitatory action in the secondary focus because of a permanent shift in the

[1]Institut de Neurobiologie de la Mediterranee (INMED)–INSERM U29, 163 Route de Luminy, Marseilles 13273, France. [2]Present address: Dartmouth Medical School, Lebanon, New Hampshire 03756, USA. Correspondence should be addressed to Y.B.-A. (ben-ari@inmed.univ-mrs.fr).

Published online 21 September 2003; doi:10.1038/nn1125

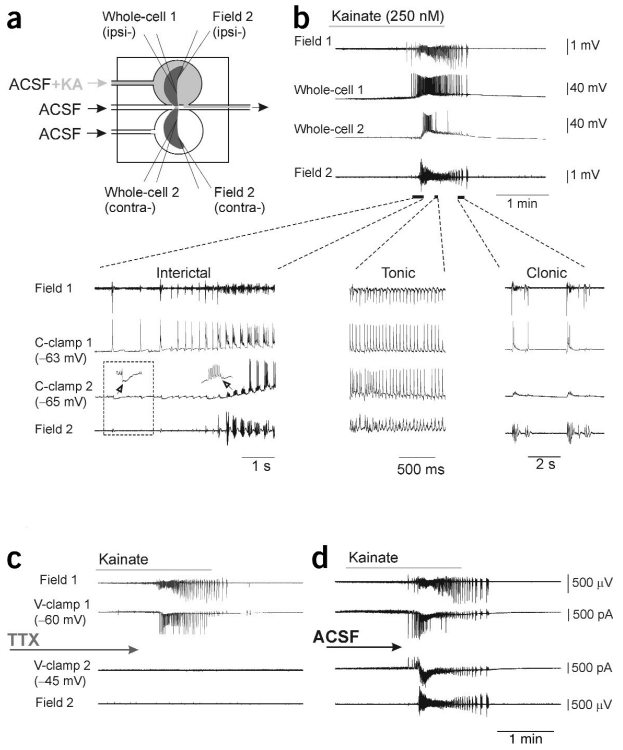

Figure 1 Interhemispheric propagation of epileptiform activity. (**a**) Schematic illustration of the experiment and of the three-compartment chamber. Two hippocampi and the commissural connections have independent inlets and a common outlet. Kainate was applied to one hippocampus (ipsi-) and electrical activity recorded in ipsilateral and contralateral (contra-) hippocampus. (**b**) Field 1 and whole-cell recordings in kainate-treated (Field 2 and Whole-cell 1) and naive (Field and Whole-cell 2) hippocampi. Expanded traces are shown below; the boxed area shows a burst of EPSCs. (**c**,**d**) Application of TTX (2 µM) to the commissural fibers blocked the interhippocampal propagation of epileptiform activity in a reversible manner.

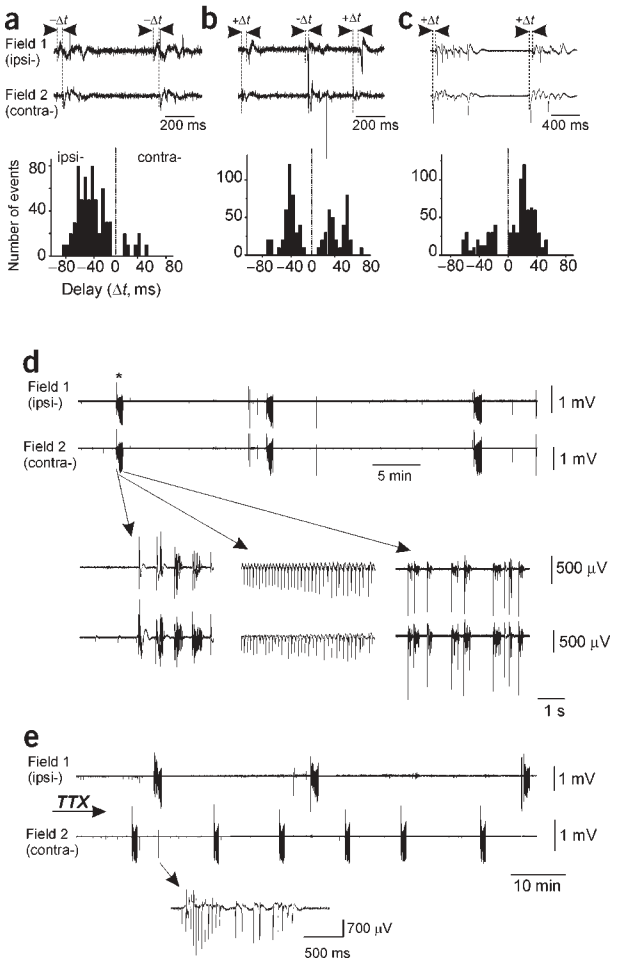

Figure 2 The naive side becomes epileptogenic after repeated seizures. Latency (Δt) between inter-hemispheric events after one (**a**) or seven (**b**) applications of kainate. After the first application of kainate, epileptiform activity originated in the treated hippocampus (**a**). In contrast, after the seventh application (**b**), epileptiform activity was equally generated in both hemispheres. (**c**,**d**) Spontaneous events were now frequently generated in the naive side. (**e**) After application of TTX to the commissural chamber, spontaneous and evoked EAs were generated in both the treated (ipsi-) and naive (contra-) hippocampus independently. Electrical stimulation of the mirror focus (arrow) generated an EA; thus the hippocampus is now epileptic.

GABA reversal potential. The formation of distal foci through seizures may lead to deleterious consequences in the developing brain by altering activity-dependent processes.

RESULTS

Repeated ictal episodes generate a secondary epileptic focus

In the intact hippocampi in a three-chamber apparatus (**Fig. 1a**), brief (2–3 min) unilateral applications of kainate (250–300 nM) generated a single episode of epileptiform activity that was composed of interictal activity (a brief rhythmic burst of population spikes) culminating in ictal seizures that propagated to the naive hippocampus (**Fig. 1b**; delay, 51.3 ± 2.7 ms; $n = 25$). A high-frequency barrage of postsynaptic potentials and action potentials was recorded in the naive hippocampus (**Fig. 1b**) after the propagation of a few epileptiform activities from the treated side. Excitatory postsynaptic currents (EPSCs) were abolished by bath application of the AMPA receptor antagonist 6-cyano-5-nitroquinoxaline-2,3-dione (CNQX; 10 µM) and the NMDA receptor agonists D-(–)-2-amino-5-phosphonovaleric acid (AP5; 50 µM; $n = 5$) or TTX (1 µM; $n = 2$; data not shown), indicating that the EPSCs may be generated by network-driven AMPA and NMDA receptors. Blocking the interhemispheric connections by applying TTX to the commissural compartment prevented the propagation of seizures from one hemisphere to the other (**Fig. 1c**). This blockade was reversible, as a third application of kainate after washout of TTX generated an epileptiform episode that did propagate to the naive hippocampus (**Fig. 1d**). Applications of kainate to the commissural chamber did not generate seizures, suggesting that kainate does not diffuse from the commissural chamber to the hippocampus, which is in keeping with earlier observations using dye applications[15,16]. Therefore, it is possible with this chamber to reversibly disconnect the two hemispheres.

Propagation of a single or a few epileptiform activities did not lead to the formation of a secondary focus in the naive side. Thus, after a single or a few epileptiform activities, there was no spontaneous epileptiform activity in the naive side, and electrical stimulation generated postsynaptic currents and field excitatory postsynaptic potentials (EPSPs) that were identical to those generated in controls (**Fig. 1c**; $n = 7$). In contrast, after 10–15 applications of kainate (with intervals of 15–20 min), the duration of each episode progressively increased (from 89.8 ± 4.6 to 117 ± 6.2 s; $n = 15$), and much epileptiform activity was now generated in the naive hippocampus and was back-propagated to

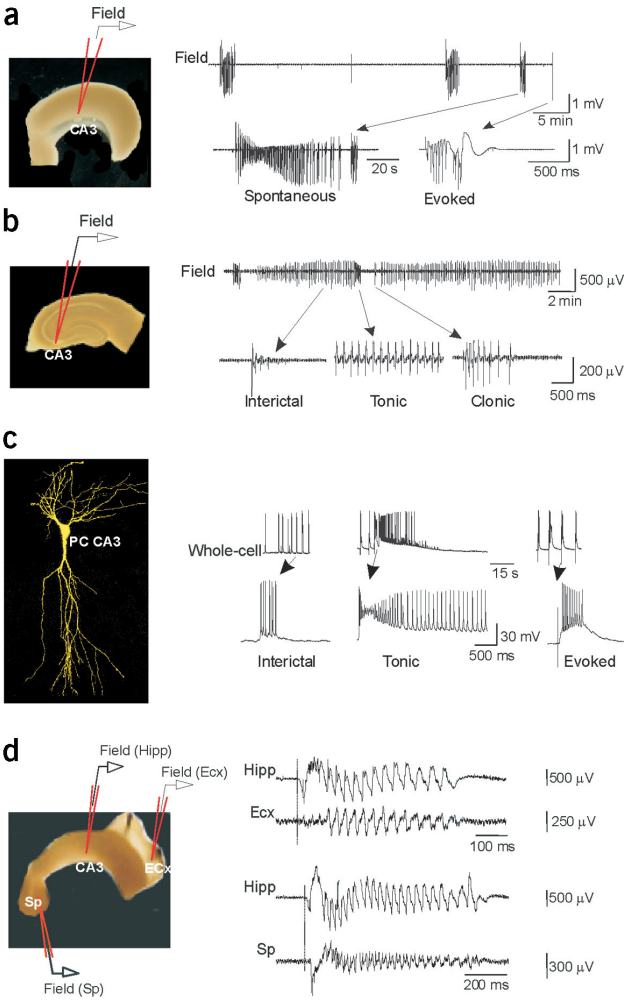

Figure 3 Chronic epileptogenesis in the secondary focus. Recordings made from the naive side 24 h after the formation of a secondary focus by repeated applications of kainate to the other hippocampus. The intact hippocampi were left in ACSF for 24 h. (**a**–**d**) The intact hippocampus (**a**), slices obtained from the intact hippocampus (**b**), CA3 pyramidal cell (PC) (**c**) filled with biocytin, and the intact hippocampus (Hipp) connected to the septal complex (Sp) and the entorhinal cortex (Ecx) (**d**). Left, pictures from the preparations (or reconstructed neuron). Right, enlargement of spontaneous and evoked epileptiform activity in **a**, epileptiform activity in a slice in **b** and spontaneous and evoked interictal bursts of action potentials in a single CA3 pyramidal neuron as recorded (whole-cell) in a slice in **c** (with an expended scale below). In **d**, paired field recordings illustrate the propagation of epileptiform activity from the hippocampus to the entorhinal cortex and septum.

the kainate-treated side. This was estimated quantitatively using incidence plots in which the delay between the epileptiform activity recorded in the two hemispheres was expressed (**Fig. 2a,b**; $n = 10$). The naive hippocampus was transformed into an epileptic structure, as spontaneous events were generated in the naïve hippocampus (**Fig. 2c,d**) electrical stimulation evoked epileptiform activity instead of the EPSP that was initially recorded (**Fig. 2d**). In addition, disconnecting the two hippocampi (by applying TTX to the commissural chamber) blocked the synchrony between the hippocampi but prevented neither the spontaneous nor the evoked seizures in the naive side in 69% of the experiments (**Fig. 2e**; $n = 42$). Therefore, repeated seizures in one hippocampus generated a secondary focus in the naive side.

The following observations indicate that propagating seizures may lead to a transformation of the naive hippocampus. First, once the mirror focus was formed, the hippocampus was disconnected from the kainate-treated side and kept *in vitro* for 1d, which allows excellent preservation of the hippocampus (data not shown). Spontaneous epileptiform activity was recorded in the intact hippocampus (**Fig. 3a**). Second, spontaneous (**Fig. 3b**) or evoked epileptiform activity (**Fig. 3c**) was recorded with field (**Fig. 3b**) and patch-clamp recordings (**Fig. 3c**) in slices prepared from the secondary focus 1 d after establishment of the focus, indicating that the 'kindling' effect may not be restricted to the intact hippocampus. Third, epileptiform activity that was generated in the secondary focus readily propagated *in vitro* to other limbic structures, including the septum and entorhinal cortex in addition to the contralateral hippocampus (**Fig. 3d**). Fourth, applications of CNQX and AP5 transiently blocked the epileptiform activity. After the antagonists were washed out, spontaneous and evoked epileptiform activity reappeared, indicating that permanent alterations may have taken place (data not shown). Therefore, repeated epileptiform activity persistently transformed a naive hippocampus into an epileptogenic structure capable of generating seizures that will propagate to other structures.

NMDA receptor activation needed for secondary focus formation

The mechanisms that lead to the formation of a secondary mirror focus require recurrent activation by seizures of neurons in the contralateral naive hippocampus. Thus, continuous applications of CNQX (20 μM; see **Supplementary Fig. 1** online) or TTX (1 μM, $n = 2$; data not shown) to the naive hippocampus, in parallel with contralateral applications of kainate, prevented both the propagation of seizures and the formation of a mirror focus. Thus, after repeated applications, spontaneous and evoked epileptiform activities were present in the kainate-treated but not in the isolated contralateral hippocampus. In contrast, when NMDA receptors were blocked in the naive side by continuous bath applications of AP5 (100 μM), seizures propagated from the treated side, but a mirror focus was not established (**Fig. 4a–c**). In the presence of AP5, repeated applications of kainate did not generate spontaneous epileptiform activity in the naive hippocampus, and the incidence plots showed a persistent generation of the epileptiform activity in the stimulated side (**Fig. 4c**; $n = 15$, up to 25 applications). Electrical stimulation evoked a field EPSP before and after repeated applications of kainate to the other hippocampus (**Fig. 4d**), and, after we blocked the interhemispheric connections by application of TTX to the commissural chamber, we did not record spontaneous seizures in the naive hippocampus (**Fig. 4e**). AP5 reduced the amplitude and duration of the epileptiform activity in the kainate-treated hippocampus but did not block the generation of spontaneous or evoked epileptiform discharges (data not shown).Therefore, the activation of NMDA receptors in the naive side is necessary for the establishment of a secondary focus but not for expression of its epileptiform activities.

Excitatory actions of GABA in the secondary focus

An important advantage of this hippocampal preparation is that it enables the determination of chronic alterations in synaptic currents that take place in a secondary focus. Because excitatory actions of GABA are important in epileptogenesis (see Discussion), we determined whether the propagation of recurrent seizures produces persistent effects on the actions of GABA. We first used noninvasive extracellular multi-unit activity (MUA) to obtain a quantitative estimate of the density of excitatory GABAergic synapses. The effects of bicuculline on MUA were compared in slices obtained from intact control and secondary foci that were kept *in vitro* for

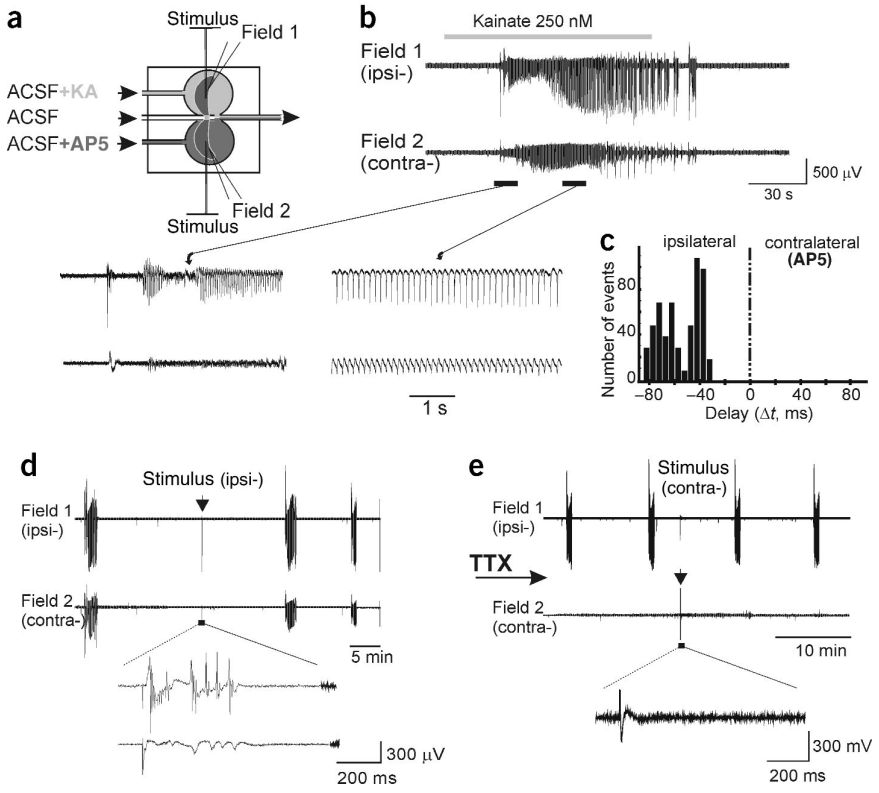

Figure 4 Activation of NMDA receptors is required for the formation of a secondary focus. (**a**) Kainate was repeatedly applied to one hippocampus (Field 1) and AP5 (100 μM) was continuously applied to the naive contralateral hippocampus (Field 2). (**b**) Field recordings indicate that epileptiform activity propagates from the treated to the naive hippocampus in spite of the continuous application of AP5 to the latter. (**c**) Interhemispheric distribution of delays in epileptiform activity after ten applications of kainate to the treated side. The seizures were always generated in the treated hippocampus. (**d**) Epileptiform activity was now spontaneously generated in the treated hippocampus and was propagated to the AP5-treated side. Stimulation of the treated hippocampus evoked epileptiform activity that propagated with attenuation to the contralateral side. (**e**) After the two hippocampi were disconnected by application of TTX to the commissural chamber, the hippocampus that was pretreated with AP5 was unable to generate spontaneous or evoked (see enlargement) epileptiform activity.

24 h before slice preparation. Bicuculline generated interictal seizures (**Fig. 5a**; $n = 12$ neurons) in control slices but blocked ongoing seizures in slices obtained from the secondary focus (**Fig. 5b**; $n = 6$ neurons).

To directly determine the excitatory actions of GABA, we measured the number of action potentials evoked by electrical stimuli in MUA recordings in the presence of glutamate receptor antagonists. AP5 (40 μM) and CNQX (20 μM) almost entirely blocked MUA in control slices but produced only a small reduction in epileptic slices (**Fig. 5c**). The remaining activity was fully blocked by bicuculline (10 μM), confirming that the spikes were generated by excitatory GABAergic synapses. Quantification of several such experiments ($n = 20$ stimuli in 6 slices) showed a higher number of bicuculline-sensitive action potentials in epileptic versus naive slices (fourfold difference; **Fig. 5d**).

Finally, we used perforated patch-clamp recordings to determine whether an alteration in the chloride reversal potential (E_{Cl}) had occurred in the secondary focus (**Fig. 6a,b**). The resting membrane potential of neurons was not different (control: 67.7 ± 0.5 mV, $n = 11$; secondary foci: 67.3 ± 0.6 mV, $n = 9$). In contrast, there was a significant shift in the reversal potential of GABA in epileptic neurons (control: 69.8 ± 2.8 mV, $n = 6$; epileptic neurons: 55.2 ± 1.9 mV, $n = 8$; $P < 0.001$). Electrical stimulation of synaptic inputs using gramicidin-perforated patch-clamp recording, in the presence of CNQX and AP5 to block glutamatergic receptors, evoked a bicuculline-sensitive burst of action potentials (**Fig. 6c**), confirming the excitatory actions of GABA ($n = 5$ neurons). Thus, GABAergic synapses were excitatory in the secondary focus, and the recurrent seizures permanently altered the developmental shift in the actions of GABA.

DISCUSSION

Seizures that propagate from one brain structure to another transform the latter to a permanent secondary epileptogenic focus. To the best of our knowledge, this is the first unequivocal description of a secondary focus in the mammalian brain *in vitro*. Relying on the intrinsic advantages offered by this preparation, in particular the possibility of preserving the secondary focus *in vitro*, we have identified some of the mechanisms involved in the induction and expression of the secondary focus. The formation of a secondary focus requires the activation of NMDA receptors, and the expression includes a shift in the reversal potential of GABA. The excitatory actions of GABA and the opposite effects of bicuculline in age-matched control and epileptic foci indicate that the excitatory actions of GABA may be of central importance in the generation of seizures. This raises the possibility that immature neurons may have a high degree of vulnerability to mechanisms that interfere with chloride efflux. Further studies are required to compare the mechanisms that regulate chloride in adult and neonatal neurons and to understand how these mechanisms are modulated by hyperactivity.

Mirror focus formation is mediated by NMDA reseptors

Seizures directly and rapidly triggered the formation of a permanent secondary focus with local recurrent spontaneous seizures that propagate to other limbic structures. The secondary focus is epileptic, as spontaneous ictal and interictal events were recorded 24 h after the induction. Spontaneous ictal events were present in slices obtained from the secondary focus, in contrast to slices obtained from human epileptic tissue or animal models of epilepsies[17–20]. Thus, the elements required for the generation of ictal events are included in a slice prepared form the secondary focus but not in slices prepared from human or animal epileptic structures.

The induction of long-term epileptiform changes in the contralateral hippocampus required the activation of NMDA receptors in the naive circuit. Seizures activate NMDA receptors in a wide range of preparations[19,21–23], and blockade of NMDA receptors prevents the long-term consequences of seizures without preventing the propagation of seizures *per se*[19,22,24]. The present study additionally shows that bath application of an NMDA receptor antagonist directly to the contralateral hippocampus prevents the formation of a secondary focus, although this does not prevent the propagation of seizures. The

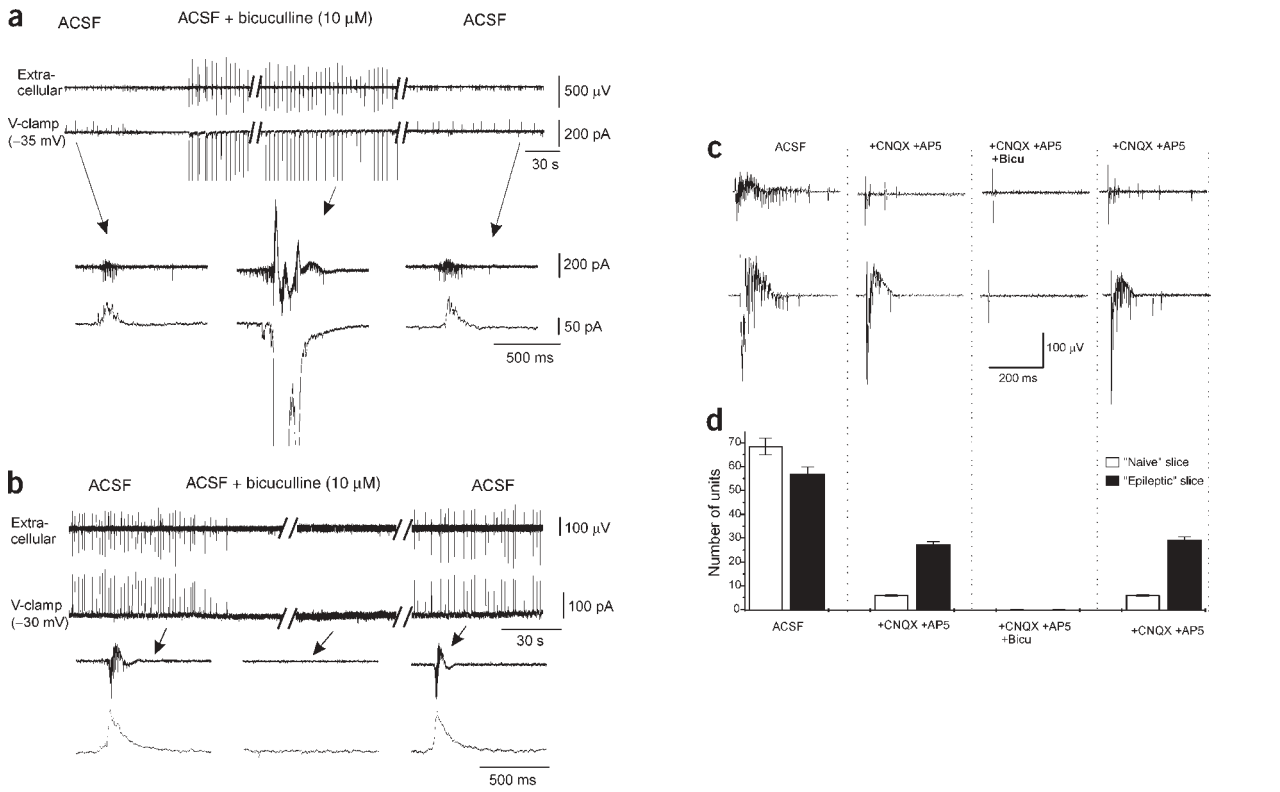

Figure 5 Different effects of bicuculline in control and epileptic slices. Age-matched (P7) intact hippocampi were kept for 24 h *in vitro*. (**a**) Bicuculline generated seizures in the slice obtained from control hippocampus, as shown by extracellular and whole-cell (V-clamp) recordings. (**b**) In a slice from the secondary focus, bicuculline blocked ongoing seizures. (**c**) In a naive slice (top) obtained from a control intact hippocampus, AP5 and CNQX almost entirely blocked MUA; the remaining activity was reversibly blocked by bicuculline (Bicu), confirming that it is generated by the excitatory action of GABA. In contrast, in an epileptic slice obtained from a secondary focus (bottom), a significant component of MUA was present during applications of AP5 and CNQX. (**d**) Quantification of the data: 20 stimuli were applied in each slice, 6 individual experiments were summed and the mean number of units evoked by a single stimulus was determined. Glutamatergic receptor antagonists blocked 90% of the MUA in controls and only 50% in epileptic slices.

sequence that underlies the formation of a secondary focus is analogous to that involved in an activity-dependent increase in synaptic efficacy. High-frequency tetanic stimuli generate long-term potentiation of synaptic transmission, an effect that requires the activation of NMDA receptors for its induction, but not for its maintenance[25]. Similarly, brief seizures in adult slices generate an epileptic long-term potentiation of synaptic transmission, as electrical stimuli delivered after the event generate an epileptic current instead of the EPSC recorded before the seizures[19]. Further studies are required to determine what changes in synaptic efficacy take place after seizures and to compare the mechanisms that produce a long-term increase of synaptic efficacy in long-term potentiation and epileptogenesis. This preparation is highly suitable for these studies.

Excitatory actions of GABA in the secondary focus

A central issue in seizure generation is the failure of inhibition and the shift in GABA action from inhibition to excitation. A reduction or loss of the efficiency of GABAergic synapses has been reported in a wide range of adult seizure models *in vitro* and *in vivo* including acute high-frequency stimulations that generate seizure[26], electrical kindling[27] and kainate or pilocarpine administration, which generates a genuine model of temporal-lobe epilepsies[2,28]. Several mechanisms underlie the loss of inhibition. These include desensitization of GABAergic currents[26]; a loss of miniature GABAergic currents and quantal release of GABA[29]; a loss of a subtype of interneurons[30–32] and a selective reduction in the frequency of dendritic inhibitory postsynaptic currents but not of somatic inhibition[32,33]. The last leads to a selective reduction in the control of dendritic inhibition and facilitates the spread of sodium and calcium currents into the soma[34]. Alterations in GABAergic currents also are important in the formation of a mirror focus *in vivo* in the cat somatosensory cortex[35].

More recently, a chronic excitatory action of GABAergic synapses has been reported in adult human temporal lobe[17]. As this change occurs in neurons that do not generate spontaneous ictal events, it is likely that a shift in E_{Cl} participates in the generation and is not merely a consequence of seizures. In all brain structures and species studied to date, higher intracellular concentrations of chloride are present in naive immature neurons than in mature neurons[13]. The activation of GABA synapses generates Na^+ and Ca^{2+} action potentials, removes the voltage-dependent Mg^{2+} block of NMDA channels and increases $[Ca^{2+}]_i$[13,36,37]. This developmental curve is largely due to a delayed expression of a KCC2 chloride extruder[38–40]. In the present study, the response evoked in both noninvasive extracellular or perforated patch-clamp recordings, which do not alter the E_{Cl}, suggest an excitatory role for GABA in the secondary focus but not in age-matched control neurons. Bicuculline generated seizures in control slices but reduced or blocked seizures generated in the secondary focus. This indicates that the E_{Cl} shift may be a fundamental feature of the focus that participates in the generation of seizures in the developing hippocampus. Although further studies are required

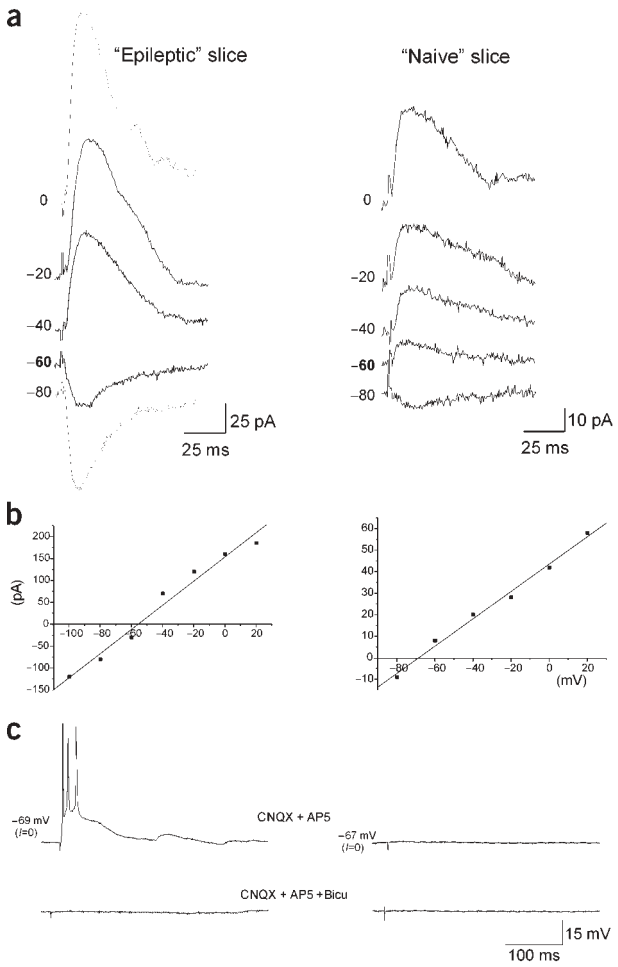

Figure 6 Shift in E_{Cl} and in the excitatory actions of GABA in the secondary focus. (**a**) Whole-cell gramicidin-perforated patch-clamp recordings of the EPSCs evoked by electrical stimulations. (**b**) Corresponding *I–V* curves show the different mean E_{Cl} in several similar experiments. Note the different reversal potential of GABAergic postsynaptic currents in control and epileptic neurons in **a** and **b**. (**c**) Excitatory actions of GABAergic postsynaptic potentials in similar perforated patch-clamp recordings carried out in the presence of CNQX and AP5 to block glutamatergic synapses. At the resting membrane potential, no current was injected ($I = 0$). An electrical stimulation evoked a burst of action potentials in the neurons in the secondary focus and no response in the control neuron. Application of bicuculline (Bicu) blocked the burst, indicating that it is mediated by the activation of GABA receptors.

to compare the effects of seizures on the E_{Cl} in immature and adult neurons, it is possible that seizures affect the developmental shift of GABA actions from excitation to inhibition, which is known to be important in epileptogenesis of the immature hippocampus[7,41,42]. The delayed expression of the chloride exporter KCC2 is largely responsible for the excitatory-inhibitory shift of GABA actions[39,40], and an immature chloride transport system underlies the low threshold for seizure generation in the developing brain[42]. As the expression of the chloride transporter, which is probably regulated by activity[39], also depends on the morphological development of neurons[40], adjacent neurons that are less developed will have a higher $[Cl^-]_i$, and the excitatory actions of GABA may directly generate seizures. Future studies will have to determine the complex relationship between reversal potential in developing neurons, hyperactivity and the deleterious consequences of seizures.

Propagation of seizures and clinical relevance

Animal models have obvious limitations when a complex situation, such as that present in the human epileptic brain, is studied. Yet, *in vitro* preparations can only partly reveal the complex events that take place *in vivo*. In spite of its advantages over other preparations, the triple chamber and secondary focus do not provide a long-term model that takes into account the alterations that occur over a period of years in the clinical situation. Nevertheless, our observations have substantial clinical implications. Newborns are prone to frequent seizures and frequently develop status epilepticus. Newborns are particularly prone to frequent electrographic seizures following brief interictal periods[43]. The dependence of the secondary focus upon NMDA receptors raises the intriguing possibility that drugs that modify NMDA receptors may be beneficial in newborns with seizures. In keeping with clinical data[44], our observations indicate that repetitive—not a single or a few—recurrent seizures may be required to trigger the formation of a secondary focus. This information may be particularly useful considering the persistent debate over the need for rapid treatment of relatively benign seizures, which involves anti-epileptic agents that exert adverse effects in immature neurons from humans and animals[7,44,45]. The intact preparation and the triple chamber provides new perspectives on *in vitro* studies into the pathogenesis and treatment of temporal-lobe epilepsy, because this method allows direct study of anti-epileptic agents. It is now possible to disconnect both hemispheres reversibly and to determine the deleterious consequences of propagated seizures directly, thus eliminating many obstacles that have hindered the study of the pathophysiological mechanisms of epileptogenesis. As such, this preparation is an ideal *in vitro* system for studying mechanisms of secondary epileptogenesis and activity-dependent neuronal plasticity.

METHODS

Review of animal experimentation. The present experiments were designed according to the regulations of the French Medical Research Council (INSERM) and with the approval of its animal care committee.

Hippocampal preparation. The interconnected intact hippocampal formation (IIHF) was prepared as previously described[15,16]. In brief, neonatal (postnatal day (P)6–7) male Wistar rats were decapitated after hypothermic anesthesia, and their brains were rapidly removed to ice-cold oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF): 126 mM NaCl, 3.5 mM KCl, 2.0 mM $CaCl_2$, 1.3 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4 and 11 mM glucose, pH 7.4. Complexes including two interconnected hippocampi were isolated and transferred to a beaker containing oxygenated ACSF and were kept there ≥1 h before use. IIHFs were placed into a conventional fully submerged three-compartment chamber that was superfused with oxygenated ACSF at 28–30 °C at a rate of 10–15 ml/min. The IIHFs were fixed to the Sylgard bottom using entomological needles.

The three-compartment chamber has two principal differences from the two-compartment chamber that was used in our previous studies[15,16]: the third compartment is introduced between the two hippocampal chambers with a groove to lodge and perfuse the interhippocampal commissure separately; and the common outlet is used for all three compartments, which prevents leakage between the chambers (**Fig. 1a**; see also ref. 15).

Electrophysiological recordings. All recordings were made in area CA3. We used the patch-clamp technique in voltage-clamp or current-clamp configurations using an Axopatch 200B (Axon Instruments) for whole-cell recordings. The whole-cell patch pipettes had a resistance of 8–10 MΩ when filled with a solution containing 135 mM K-gluconate, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Mg-ATP, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-

ethanesulfonic acid (HEPES), pH 7.25 (osmolarity, 270 mosM). We identified cells by adding lucifer yellow (0.5%) and/or biocytin (0.4%) to the pipette solution for morphological analysis.

The patch-pipette solution for gramicidin-perforated patch recording contained 150 mM KCl and 10 mM HEPES, buffered to pH 7.2 with Tris-OH. Gramicidin was first dissolved in DMSO to prepare a stock solution of 40 mg/ml and then was diluted to a final concentration of 80 μg/ml in the pipette solution. The gramicidin-containing solution was prepared 30 min before the experiment. Patch pipettes were filled with this gramicidin-containing solution, and then the tips of the pipettes were dipped into and filled with a gramicidin-free solution by applying negative pressure for 30–40 s to facilitate cell-attached formation (4–10 GΩ). Series resistance decreased and stabilized 20–30 min after a cell attachment was formed. At the end of each recording, we applied negative pressure to break the membrane and establish a whole-cell configuration. This was associated with a shift in the reversal potential of the $GABA_A$-mediated responses to ~0 mV.

We recorded extracellular potentials using two types of electrode. For field recordings, we used glass micropipettes (1.2 mm o.d. × 0.94 mm i.d.; GC120TF-10, Clark Electromedical Instruments) filled with ACSF (1–5 MΩ). To record MUA, we used tungsten wire electrodes (diameter, 50 μm; California Fine Wire). Recording electrodes were positioned in a subfield of pyramidal cell layer CA3 and signals were amplified using a custom-made DAM-80i amplifier (WPI; low filter, 5 Hz; high filter, 3 KHz; ×1,000). Electrical stimulations were done with a bipolar electrode (10–20 V, 40 μs).

Synaptic responses were acquired into the memory of a personal computer using an analog-to-digital converter (Digidata 1200; Axon Instruments). We used Axoscope 7.0 (Axon Instruments), Acquis (Gerard Sadoc) and Origin 5.0 (Microcal Software) for the acquisition and analysis of the synaptic activities. Group measures are expressed as means ± s.e.m.; error bars also indicate s.e.m. We assessed the statistical significance of differences with the Student's *t*-test; the level of significance was set at $P < 0.05$.

Drugs used were purchased from Sigma (TTX, gramicidin D), Tocris Neuramin (bicuculline, CNQX, AP5) and Molecular Probes (lucifer yellow, biocytin).

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We are indebted for financial support to the Institut de la Santé et de la Recherche Médicale (INSERM), the French foundation of medial research, the Cino Del Duca and the Electricité de France foundations. I.K. received financial support from the French Federation of Research in Epilepsies, INSERM and International Brain Research Organization. We thank R. Khazipov for his suggestions and criticism.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 27 June; accepted 28 July 2003
Published online at http://www.nature.com/natureneuroscience/

1. Moshe, S.L. & Albala, B.J. Kindling in developing rats: persistence of seizures into adulthood. *Brain Res.* **256**, 67–71 (1982).
2. Ben-Ari, Y. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience* **14**, 375–403 (1985).
3. Cendes, F. *et al.* Frequency and characteristics of dual pathology in patients with lesional epilepsy. *Neurology* **45**, 2058–2064 (1995).
4. Wieser, H.G., Ortega, M., Friedman, A. & Yonekawa, Y. Long-term seizure outcomes following amygdalohippocampectomy. *J. Neurosurg.* **98**, 751–763 (2003).
5. Hauser, W.A. Epidemiology of epilepsy in children. *Neurosurg. Clin. N. Am.* **6**, 419–429 (1995).
6. Moshe, S.L., Albala, B.J., Ackermann, R.F. & Engel, J. Jr. Increased seizure susceptibility of the immature brain. *Brain Res.* **283**, 81–85 (1983).
7. Holmes, G.L. & Ben-Ari, Y. Seizures in the developing brain: perhaps not so benign after all. *Neuron* **21**, 1231–1234 (1998).
8. Chen, K., Baram, T.Z. & Soltesz, I. Febrile seizures in the developing brain result in persistent modification of neuronal excitability in limbic circuits. *Nat. Med.* **5**, 888–894 (1999).
9. Villeneuve, N., Ben-Ari, Y., Holmes, G.L. & Gaiarsa, J.L. Neonatal seizures induced persistent changes in intrinsic properties of CA1 rat hippocampal cells. *Ann. Neurol.* **47**, 729–738 (2000).
10. Komuro, H. & Rakic, P. Modulation of neuronal migration by NMDA receptors. *Science* **260**, 95–97 (1993).
11. Constantine-Paton, M., Cline, H.T. & Debski, E. Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways. *Annu. Rev. Neurosci.* **13**, 129–154 (1990).
12. Barbin, G., Pollard, H., Gaiarsa, J.L. & Ben-Ari, Y. Involvement of $GABA_A$ receptors in the outgrowth of cultured hippocampal neurons. *Neurosci. Lett.* **152**, 150–154 (1993).
13. Ben Ari, Y. Excitatory actions of gaba during development: the nature of the nurture. *Nat. Rev. Neurosci.* **3**, 728–739 (2002).
14. Raymond, A.A. *et al.* Association of hippocampal sclerosis with cortical dysgenesis in patients with epilepsy. *Neurology* **44**, 1841–1845 (1994).
15. Khalilov, I. *et al.* A novel in vitro preparation: the intact hippocampal formation. *Neuron* **19**, 743–749 (1997).
16. Khazipov, R., Desfreres, L., Khalilov, I. & Ben-Ari, Y. Three-independent-compartment chamber to study in vitro commissural synapses. *J. Neurophysiol.* **81**, 921–924 (1999).
17. Cohen, I., Navarro, V., Clemenceau, S., Baulac, M. & Miles, R. On the origin of interictal activity in human temporal lobe epilepsy in vitro. *Science* **298**, 1418–1421 (2002).
18. Louvel, J. *et al.* GABA-mediated synchronization in the human neocortex: elevations in extracellular potassium and presynaptic mechanisms. *Neuroscience* **105**, 803–813 (2001).
19. Ben-Ari, Y. & Gho, M. Long-lasting modification of the synaptic properties of rat CA3 hippocampal neurones induced by kainic acid. *J. Physiol* **404**, 365–384 (1988).
20. Esclapez, M., Hirsch, J.C., Ben-Ari, Y. & Bernard, C. Newly formed excitatory pathways provide a substrate for hyperexcitability in experimental temporal lobe epilepsy. *J. Comp Neurol.* **408**, 449–460 (1999).
21. Rafiq, A., DeLorenzo, R.J. & Coulter, D.A. Generation and propagation of epileptiform discharges in a combined entorhinal cortex/hippocampal slice. *J. Neurophysiol.* **70**, 1962–1974 (1993).
22. Stasheff, S.F., Anderson, W.W., Clark, S. & Wilson, W.A. NMDA antagonists differentiate epileptogenesis from seizure expression in an in vitro model. *Science* **245**, 648–651 (1989).
23. Neuman, R., Cherubini, E. & Ben-Ari, Y. Epileptiform bursts elicited in CA3 hippocampal neurons by a variety of convulsants are not blocked by *N*-methyl-D-aspartate antagonists. *Brain Res.* **459**, 265–274 (1988).
24. Trommer, B.L. & Pasternak, J.F. NMDA receptor antagonists inhibit kindling epileptogenesis and seizure expression in developing rats. *Brain Res. Dev. Brain Res.* **53**, 248–252 (1990).
25. Malenka, R.C. & Nicoll, R.A. NMDA-receptor-dependent synaptic plasticity: multiple forms and mechanisms. *Trends Neurosci.* **16**, 521–527 (1993).
26. Ben-Ari, Y., Reinhardt, W. & Krnjevic, K. Lability of synaptic inhibition of hippocampal pyramidal cells. *Can. J. Physiol. Pharmacol.* **57**, 1462–1466 (1979).
27. Mody, I. & Heinemann, U. NMDA receptors of dentate gyrus granule cells participate in synaptic transmission following kindling. *Nature* **326**, 701–704 (1987).
28. Lemos, T. & Cavalheiro, E.A. Status epilepticus and the late development of spontaneous seizures in the pilocarpine model of epilepsy. *Epilepsy Res. Suppl.* **12**, 137–144 (1996).
29. Hirsch, J.C. *et al.* Deficit of quantal release of GABA in experimental models of temporal lobe epilepsy. *Nat. Neurosci.* **2**, 499–500 (1999).
30. Sloviter, R.S. Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science* **235**, 73–76 (1987).
31. Houser, C.R. & Esclapez, M. Vulnerability and plasticity of the GABA system in the pilocarpine model of spontaneous recurrent seizures. *Epilepsy Res.* **26**, 207–218 (1996).
32. Esclapez, M., Hirsch, J.C., Khazipov, R., Ben-Ari, Y. & Bernard, C. Operative GABAergic inhibition in hippocampal CA1 pyramidal neurons in experimental epilepsy. *Proc. Natl. Acad. Sci. USA* **94**, 12151–12156 (1997).
33. Cossart, R. *et al.* Dendritic but not somatic GABAergic inhibition is decreased in experimental epilepsy. *Nat. Neurosci.* **4**, 52–62 (2001).
34. Miles, R., Toth, K., Gulyas, A.I., Hajos, N. & Freund, T.F. Differences between somatic and dendritic inhibition in the hippocampus. *Neuron* **16**, 815–823 (1996).
35. Szente, M.B. & Boda, B. Cellular mechanisms of neocortical secondary epileptogenesis. *Brain Res.* **648**, 203–214 (1994).
36. Ben-Ari, Y., Cherubini, E., Corradetti, R. & Gaiarsa, J.L. Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J. Physiol.* **416**, 303–325 (1989).
37. Leinekugel, X., Medina, I., Khalilov, I., Ben-Ari, Y. & Khazipov, R. Ca^{2+} oscillations mediated by the synergistic excitatory actions of $GABA_A$ and NMDA receptors in the neonatal hippocampus. *Neuron* **18**, 243–255 (1997).
38. Rivera,C. *et al.* The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* **397**, 251–255 (1999).
39. Ganguly, K., Schinder, A.F., Wong, S.T. & Poo, M. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell* **105**, 521–532 (2001).
40. Wang, C. *et al.* Developmental changes in KCC1, KCC2, and NKCC1 mRNA expressions in the rat brain. *Brain Res. Dev. Brain Res.* **139**, 59–66 (2002).
41. Khalilov, I., Dzhala, V., Ben-Ari, Y. & Khazipov, R. Dual role of GABA in the neonatal rat hippocampus. *Dev. Neurosci.* **21**, 310–319 (1999).
42. Dzhala, V.I. & Staley, K.J. Excitatory actions of endogenously released GABA contribute to initiation of ictal epileptiform activity in the developing hippocampus. *J. Neurosci.* **23**, 1840–1846 (2003).
43. Clancy, R.R. & Legido, A. The exact ictal and interictal duration of electroencephalographic neonatal seizures. *Epilepsia* **28**, 537–541 (1987).
44. Shinnar, S. *et al.* The risk of seizure recurrence after a first unprovoked afebrile seizure in childhood: an extended follow-up. *Pediatrics* **98**, 216–225 (1996).
45. Bittigau, P. *et al.* Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proc. Natl. Acad. Sci. USA* **99**, 15089–15094 (2002).