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Epileptogenic Actions of GABA and Fast Oscillations in the Developing Hippocampus

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Summary

GABA excites immature neurons and inhibits adult ones, but whether this contributes to seizures in the developing brain is not known. We now report that in the developing, but not the adult, hippocampus, seizures beget seizures only if GABAergic synapses are functional. In the immature hippocampus, seizures generated with functional GABAergic synapses include fast oscillations that are required to transform a naive network to an epileptic one: blocking GABA receptors prevents the long-lasting sequels of seizures. In contrast, in adult neurons, full blockade of GABA(A) receptors generates epileptogenic high-frequency seizures. Therefore, purely glutamatergic seizures are not epileptogenic in the developing hippocampus. We suggest that the density of glutamatergic synapses is not sufficient for epileptogenesis in immature neurons; excitatory GABAergic synapses are required for that purpose. We suggest that the synergistic actions of GABA and NMDA receptors trigger the cascades involved in epileptogenesis in the developing hippocampus.

Introduction

The incidence of epilepsies is highest early in life in humans (Holmes and Ben-Ari, 1998), and neonatal animals have a higher susceptibility to epileptogenic agents or procedures (Tremblay et al., 1984; Holmes et al., 1984). The mechanisms underlying this developmental susceptibility are not understood. One candidate mechanism is the developmental shift of the actions of GABA in cortical circuits: GABAergic synapses provide most of the inhibitory drive of adult networks yet excite immature networks due to a different $[Cl^-]_i$ (Ben-Ari et al., 1997; Ben-Ari, 2002). In immature neurons, the activation of GABAergic synapses generates sodium and calcium action potentials and removes the voltage-dependent Mg^{2+} blockade from NMDA receptors, thereby enabling a synergistic action of GABA and NMDA receptors (Leinekugel et al., 1997; Ben-Ari et al., 1997). Since GABAergic synapses are also formed before glutamatergic ones in the hippocampus, GABA provides most of the excitatory drive at an early developmental stage (Ben-Ari, 2002; Ben-Ari et al., 2004) and may therefore contribute actively to generate seizures (Dzhala and Staley, 2003). The intracellular chloride concentration falls, and GABA resumes its classical inhibitory function by the end of the first postnatal week in rats (Ben-Ari, 2002) and in utero in macaque (Khazipov et al., 2001), and thus most likely also in utero in humans. The first aim of the present study was to investigate the role of GABA receptors in seizure generation—ictogenesis—in the developing hippocampus.

Epilepsy is a chronic disease manifested by the generation of spontaneous recurring seizures. Whereas ictogenesis has been extensively studied, the mechanisms of epileptogenesis—the transformation of a naive network to one that generates seizures—are poorly understood. Although "seizures beget seizures" (Gowers, 1901; Khalilov et al., 2003), we do not know why some recurrent seizures permanently transform a naive network into an epileptic one whereas others do not. Experimental evidence suggests that a strong excitatory drive—generated by glutamatergic synapses—produces an NMDA receptor-dependent increase of synaptic efficacy, leading to a reduction of the threshold of seizure generation (Ben-Ari and Gho, 1988; Stasheff et al., 1989). Since GABAergic synapses excite immature neurons and provide a significant proportion of the total excitatory drive, we have hypothesized that functional GABAergic synapses are required for epileptogenesis to occur in the immature, but not in the adult, brain.

We have now determined the roles of GABAergic synapses in ictogenesis and epileptogenesis of the developing hippocampus. We have used a preparation composed of a three-compartment chamber that accommodates the two intact hippocampi and their connecting fibers (Khazipov et al., 1999; Khalilov et al., 1997, 2003). We report that although GABA(A) receptor antagonists are ictogenic in the developing hippocampus, recurrent seizures generated without functional GABAergic synapses *do not* result in later development of spontaneous seizures, suggesting that GABAergic synapses are required for epileptogenesis. Seizures generated without operative GABA synapses do not contain very fast oscillations (FOs) that are instrumental for epileptogenesis. Therefore, GABAergic interneurons and the network-driven oscillations that they generate (Bragin et al., 1995, Whittington et al., 1995, 1997; Traub et al., 1996, 1998, 2005; Csicsvari et al., 2003; Whittington and Traub, 2003; Buzsaki and Draguhn, 2004; Fisahn et al., 1998, 2004; Somogyi and Klausberger, 2005) are necessary for seizures to beget seizures in the immature network.

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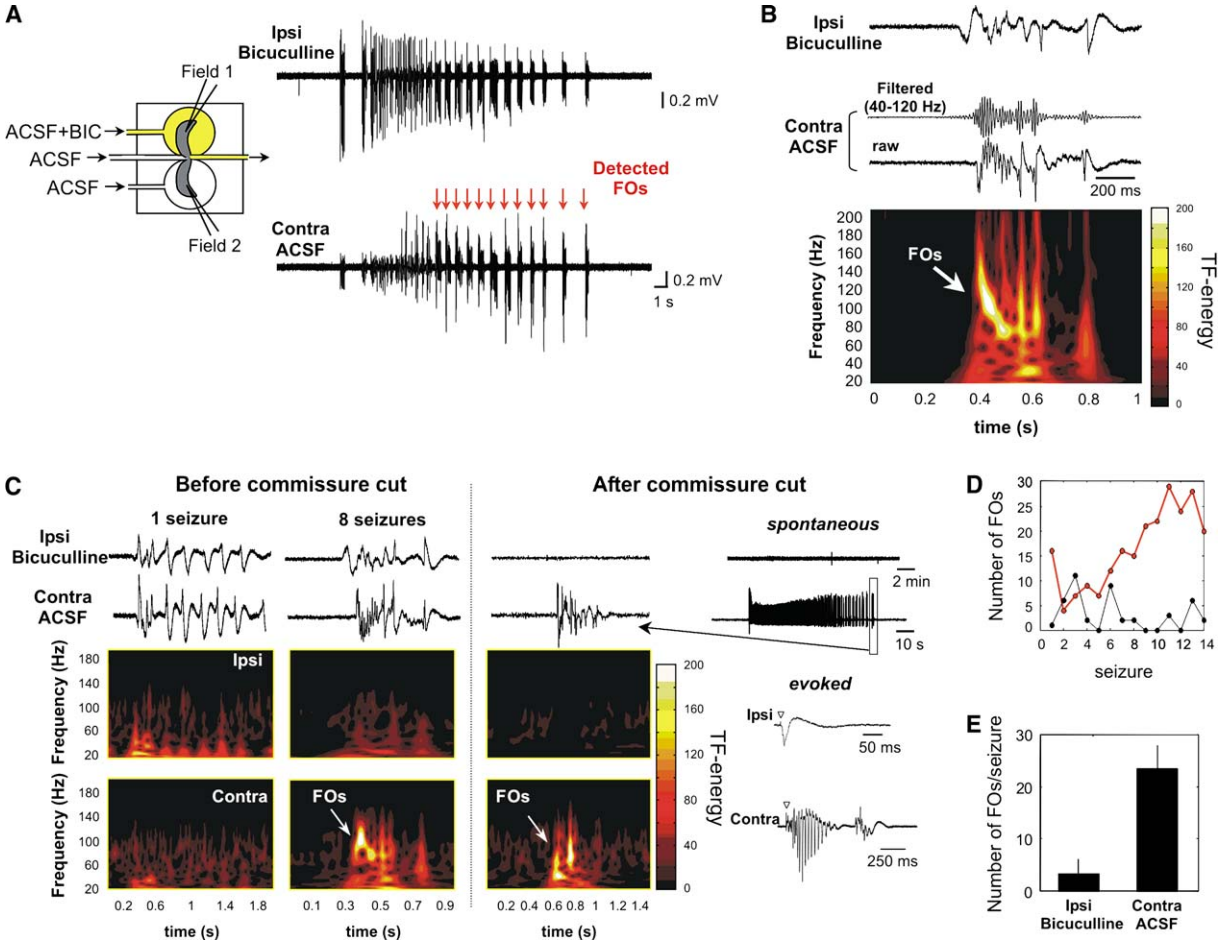

Figure 1. Activation of GABA(A) Receptors Is Required for the Generation of FOs and the Formation of a Secondary Focus

(A) Scheme of the experimental three-compartment chamber (left). Two hippocampi and the commissural connections have independent inlets and a common outlet, allowing independent perfusion of each hippocampus and commissure. Bicuculline was applied to the ipsilateral compartment (ipsi), whereas the contralateral compartment (contra) was perfused with normal ACSF. Seizures induced in the ipsilateral hippocampus propagate to the contralateral one (left). Fast Oscillations (FOs; 40–120 Hz, red arrows) were observed mainly in the contralateral hippocampus.

(B) These high-frequency activities were visualized by band-pass filtering and by time-frequency (TF) representations. The field power is here coded in colors so that white corresponds to higher amplitudes.

(C) After the propagation of a few epileptiform activities, γ activities are present in the naive contralateral hippocampus with comparable frequency and duration, as shown by the TF representations. After 15 unilateral applications of bicuculline and when the two hippocampi were disconnected, ictal events that include FOs occurred spontaneously in the contralateral hippocampus, but not in the ipsilateral one, indicating the formation of a mirror focus. Electrical stimulation of the contralateral hippocampus (see enlargement) also generated an epileptic activity.

(D) In the contralateral hippocampus where a mirror focus was generated, the number of FOs gradually increased (red line). In contrast, their number remained low in the ipsilateral hippocampus.

(E) Quantification of several experiments (n = 11) showed a higher number of FOs in the contralateral hippocampus in which GABA receptors were not blocked. Values represent the mean ± SEM.

Results

GABAergic Synapses Are Required for Epileptogenesis in Immature Neurons

To study epileptogenesis in the developing hippocampus, we used a preparation composed of a triple chamber that accommodates the two intact hippocampi and their commissural connections in three independent compartments (Khazipov et al., 1999; Khalilov et al., 1999a, 2003). In this preparation, which is restricted to immature hippocampi, it is possible to apply a convulsive agent to one hippocampus, record the propagation of seizures to the other naive hippocampus, separate the two hippocampi, and determine whether these events have led to the transformation of the naive contralateral hippocampus to an epileptogenic mirror focus that generates spontaneously epileptiform activity (Khalilov et al., 2003). Using this preparation, we showed that the propagation of a few seizures from the kainate-treated hippocampus to the naive side is sufficient to generate a chronic epileptic focus (Khalilov et al., 2003). Thus, seizures trigger the formation of an epileptic mirror focus.

We first tested whether functional GABAergic synapses are required for the formation of a mirror focus. Applications of GABA(A) receptor antagonists (bicuculline, 20 μM, n = 8; or gabazine, 10 μM, n = 3) to one hippocampus generated seizures that propagated to the opposite hippocampus (Figure 1A). Repeated applications of the

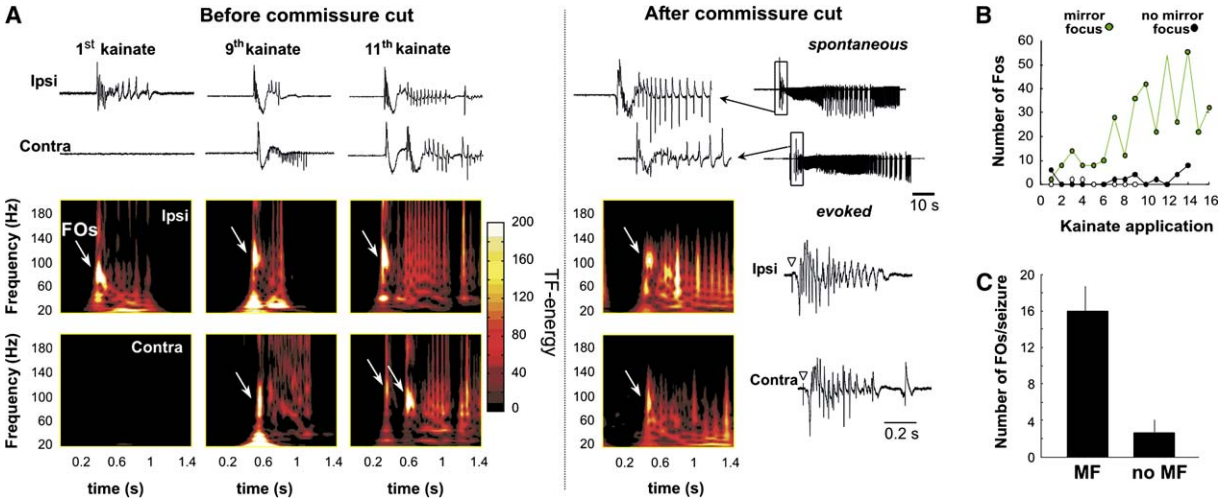

Figure 2. Kainate-Induced FOs Are Associated with Mirror Focus Formation

(A) Kainate (250–300 nM) was repeatedly applied to one hippocampus (ipsi), whereas the contralateral compartment (contra) was perfused with normal ACSF. After the propagation of a few epileptiform activities, FOs were present in the naive contralateral hippocampus with comparable frequency and duration, as shown by the TF representations. After 14 unilateral applications of kainate and when the two hippocampi were disconnected, ictal events with FOs occurred spontaneously on both sides, indicating the formation of a mirror focus. Electrical stimulation (see enlargement) also generated an epileptic activity in both hippocampi.

(B) When a mirror focus was generated (as shown in [A]), the number of FOs gradually increased in the contralateral hippocampus with repeated kainate applications. In some experiments, the mirror focus failed to form (black line) and the number of FOs remained low.

(C) Quantification of several experiments showed a higher number of FOs in the case of a mirror focus formation. Values represent the mean ± SEM.

antagonists (up to 15 applications) led to the formation of a mirror focus since spontaneous and evoked seizures were observed when the hippocampus was disconnected from the treated hippocampus (Figures 1A and 1C). However, unexpectedly, the bicuculline- or gabazine-treated ipsilateral hippocampus did not become epileptic: once the commissural connections were cut, this hippocampus generated neither spontaneous nor evoked seizures (Figure 1C). Therefore, GABA receptor antagonists are ictogenic, but not epileptogenic, in the developing hippocampus. Operative GABA synapses are needed for seizures to beget seizures.

These observations suggest a fundamental difference between seizures that include functional GABAergic synapses and those that do not. We therefore compared the properties of the seizures generated in the two hippocampi using a time-frequency (TF) analysis that enables one to determine the implication of different frequency ranges within the epileptiform activity (Thakor and Tong, 2004). A consistent difference between the seizure patterns in the two hippocampi was that the target hippocampus, but not the GABA receptor antagonist-treated hippocampus, included both γ band oscillations (40–80 Hz) and fast oscillations (from 80–140 Hz, with an average peak of 76 Hz ± 2 Hz) that are higher than the physiological γ oscillations recorded in the behaving rat (see Bragin et al., 1995; Csicsvari et al., 2003; Buzsaki and Draguhn, 2004). The mean duration of the oscillations in the target hippocampus was 120.0 ± 4.3 ms (mean ± SEM, 63 ictal events; Figures 1B and 1C). We shall refer to these patterns in general as Fast Oscillations (FOs), in keeping with earlier studies (Traub et al., 2001). Seizures generated when GABAergic synapses are blocked include only low-frequency events (19.4 ± 0.9 Hz; 63 ictal events; Figure 1C). This difference was preserved when the two hippocampi were disconnected. Furthermore, the number of FOs increased with recurrent seizures in the contralateral, but not in the local, hippocampus (Figure 1D), suggesting a build up of FOs containing seizures that led to the formation of the mirror focus. Therefore, seizures generated in the immature hippocampus without functional GABAergic synapses have a lower dominant frequency than those generated with a functional ensemble of GABAergic synapses. Seizures are required for the generation of FOs without which "seizures do not beget seizures" (Gowers, 1901).

We then tested whether the powerful convulsive agent kainate, which has been previously shown to generate a mirror focus in the same preparation (Khalilov et al., 2003), also generates FOs. Repeated applications of kainate to one hippocampus transformed both the treated and the contralateral hippocampi to chronic epileptic foci after repeated applications (Khalilov et al., 2003). With a time-frequency analysis, we observed that kainate (250–300 nM) generates a single ictal event containing FOs (~60–140 Hz, Figure 2) with an average peak of 84 ± 1 Hz and a mean duration of 125.0 ± 3.7 ms (n = 9, of nine independent preparations). FOs were concentrated at the beginning and the end of the epileptic discharge (23.0 ± 1.8 FOs/seizure; n = 9; 121 ictal events). These seizures propagated to the untreated contralateral hippocampus (Khalilov et al., 2003). With repeated applications of kainate, FOs were recorded in the untreated hippocampus (Figure 2A). Their number increased with each kainate exposure (mean increase ratio of 9.2 after ten kainate applications, relative to the first one; mean 33 FOs/seizure after ten kainate applications;

n = 9; Figure 2B). At that stage, disconnecting the two hippocampi by applications of TTX to the connecting chamber or by cutting the commissural fibers revealed the formation of a secondary mirror focus (see also Khalilov et al., 2003) and independent FOs in both hippocampi (Figure 2A). The importance of FOs in the formation of a mirror focus is suggested by the following observations. First, FOs were observed in all of the experiments in which a mirror focus was formed (n = 30/37); in the remaining experiments (n = 7/37), they were not generated by kainate and a mirror focus was not established (Figure 2). Second, in keeping with our earlier study (Khalilov et al., 2003), conditions that prevent the formation of a mirror focus also prevent the occurrence of FOs: thus, applications of kainate (up to 20 applications) to one hippocampus and the NMDA receptor antagonist APV to the other prevented the formation of a mirror focus (n = 4). Time-frequency analysis revealed that APV also prevented the generation of FOs: only low-frequency events (average peak of 9.6 ± 1.0 Hz; 24 ictal events; n = 3) were recorded in the APV-treated hippocampus (see Figure S1A in the Supplemental Data available with this article online), revealing a decrease of 98% of the number of FOs in the naive relative to the kainate-treated hippocampus ($p < 0.01$, t test; n = 3) (Figure S1B). Third, in parallel experiments, we tested the effects of repetitive electrical stimuli at high frequencies (60 Hz) or lower frequencies (10 Hz) on the induction of epileptogenesis. We found that high frequencies (n = 5), but not lower frequencies, led to a transformation of the naive network to an epileptic one (Figure S2). Therefore, FOs are instrumental in the formation of a mirror focus by kainate. Functional NMDA receptors are required for the generation of FOs, suggesting that the synergistic actions of GABA and NMDA receptors in the developing brain (Leinekugel et al., 1997; Ben-Ari et al., 1997) generate the excitatory drive that triggers the long-lasting consequences of seizures.

GABA Receptor Antagonists Are Ictogenic but Antiepileptogenic in Immature Neurons

If GABAergic synapses contribute the formation of a mirror focus, then blocking GABA receptors should also prevent the epileptic action of convulsive agents. Kainate was applied repeatedly to one hippocampus and bicuculline (20 μM, n = 5) to the contralateral side. FOs containing ictal events were recorded in the kainate-treated hippocampus (average peak of 81 ± 1 Hz) but not in the bicuculline-treated hippocampus in which low-frequency bursts were recorded (average peak of 19.4 ± 0.9 Hz; 63 ictal events; decrease of 79% relative to nontreated hippocampus; $p < 0.01$; n = 5; Figures 3A and 3B). When the two hippocampi were disconnected, the kainate-treated, but not the GABA receptor antagonist-treated, hippocampus became epileptic (Figure 3A). The reverse experiments led to similar results. When bicuculline was coapplied with kainate, FOs were present in the contralateral untreated hippocampus, but not in the kainate- and bicuculline-treated hippocampus (n = 3; Figures 3C and 3D). Spontaneous seizures developed in the contralateral hippocampus (mirror focus) but not in the kainate- and bicuculline-treated hippocampus. Thus, blocking GABA(A) receptors generated seizures but prevented the epileptogenic actions of kainate, suggesting that GABAergic synapses exert a proepileptic action in the immature brain.

To determine whether the prolonged applications of a GABA receptor antagonist do not induce nonselective effects on the network, we conducted the following experiment (Figure 4). Kainate and bicuculline were applied as above to the two hippocampi. Then, once a mirror focus was not obtained in spite of repeated (up to 15) applications of kainate to one hippocampus, the operation was repeated while applying kainate to one hippocampus and control ASCF to the other. Recurrent applications of kainate led to the formation of a mirror epileptogenic focus in the other hippocampus (n = 3). Therefore, operative GABAergic synapses are needed for seizures to beget seizures, and the actions of prolonged applications of GABA receptor antagonists remain selective.

Since GABA excites a minority of neurons at P6–P8 (Tyzio et al., 2003), we used cell-attached recordings to compare the GABA excitatory drive in the control hippocampus and the mirror focus. In the presence of glutamate receptor antagonists (CNQX, 10 μM and APV, 40 μM), an electrical stimulation generated <0.5 action potentials (mean, 0.38 ± 0.1, n = 39) in controls and >1 (mean, 1.4 ± 0.3, n = 14) in the mirror focus (Figure S3). Therefore, in keeping with earlier studies, the excitatory action of GABA is augmented in epileptic neurons.

GABAergic Synapses Are Not Required for Epileptogenesis in Adult Neurons

Are these conclusions valid for adult hippocampi? As the intact hippocampus preparation is limited to the developing hippocampus, we had to rely on another approach to determine the epileptogenic actions of GABA receptor antagonists in immature and adult hippocampi. We chose for this purpose a protocol similar to that used by Ben-Ari and Gho (1988). In adult slices, a brief application of kainate persistently transforms the naive hippocampus to an epileptogenic one. Similar to what we reported with the triple chamber, the mechanism of this transformation also involves a long-term alteration of synaptic efficacy that requires functional NMDA receptors (Ben-Ari and Gho, 1988). To directly compare adult and immature networks, we placed an adult and a neonatal slice in the same chamber (Figure 5). Applications of gabazine or bicuculline (10 μM, 10 min) were ictogenic in both adult and immature hippocampi, as expected from this and earlier studies (C. Psarropoulou and S. Descombes, 1998, Soc. Neurosci., abstract) (Borck and Jefferys, 1999; Karnup and Stelzer, 2001; Avoli, 2000). However, the agents were epileptogenic in adult, but not in immature, slices (Figure 5, n = 9). Indeed, hours after GABA(A) receptor antagonist washout, the adult, but not the neonatal, hippocampus generated spontaneous and evoked seizures, suggesting that operative GABAergic synapses are required for epileptogenesis in the immature, but not in the adult, hippocampus. A time-frequency analysis revealed that immature slices, in agreement with the observations made in the intact hippocampus, generate only low-frequency events (22 ± 2.5 Hz). In contrast, in adult slices, FOs (225 ± 5.1 Hz) were recorded in the presence of GABA(A) receptor antagonists (Figure 5).

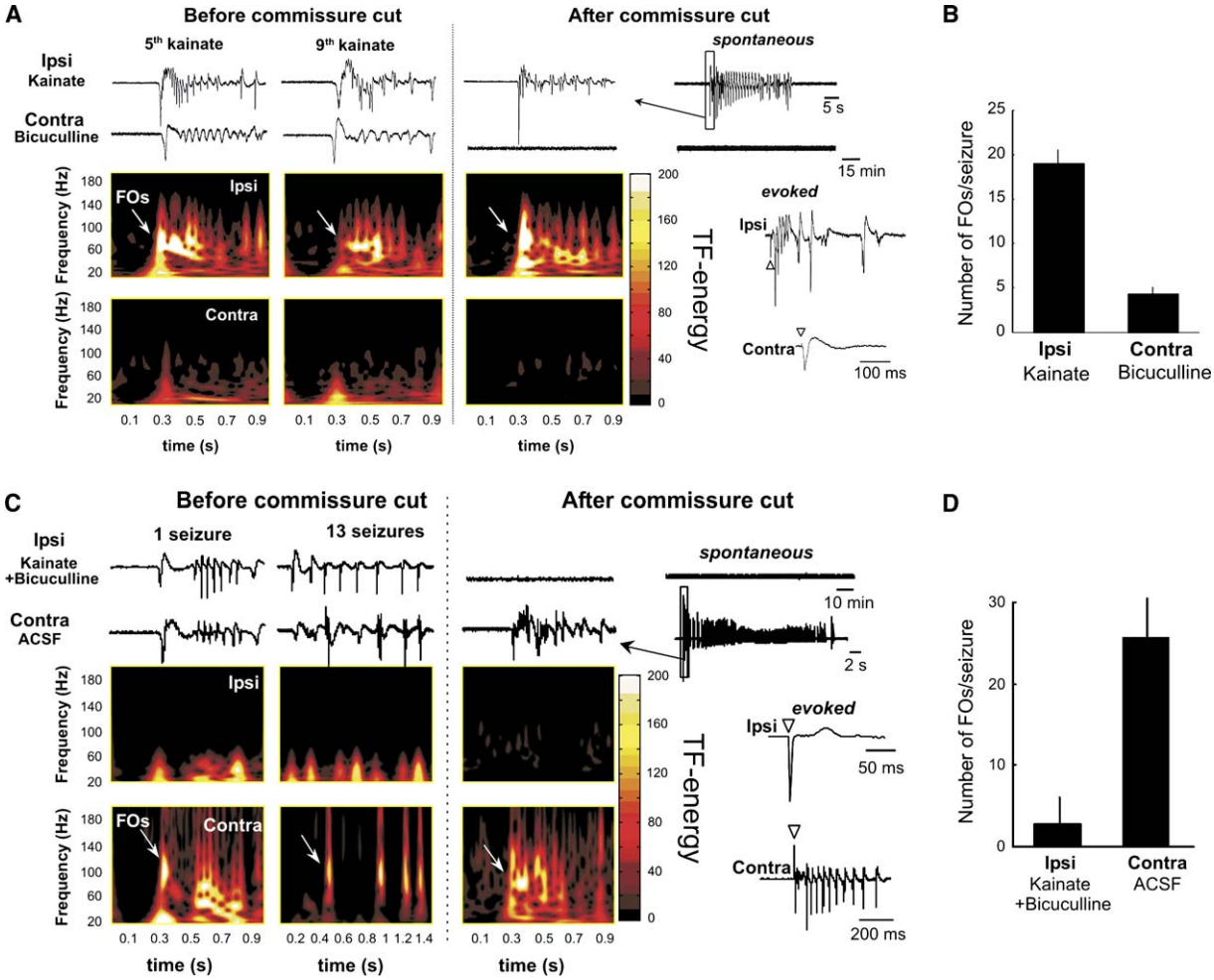

Figure 3. Blocking GABA Receptors Prevents the Epileptogenic but Not the Ictogenic Action of Kainate

(A) Kainate was applied repeatedly to one hippocampus and the GABA-A receptor antagonist bicuculline (20 μM) to the other. Bicuculline did not block the propagation of ictal-like events, but prevented both the propagation of FOs and the formation of a mirror focus. When the two hippocampi were disconnected, the primary, but not the secondary, site became epileptic, as shown by spontaneous or evoked seizures. (B) The averaged number of FOs was significantly lower in the bicuculline-treated hippocampus. (C) Kainate was applied repeatedly to one hippocampus in the presence of the GABA(A) receptor antagonist (bicuculline, 20 μM). The contralateral hippocampus was perfused with normal ACSF. FOs were present only in the contralateral hippocampus in which GABA receptors were not blocked. (D) The average number of FOs in ipsilateral and contralateral hippocampi. Values represent the mean ± SEM.

To further examine the role of GABAergic synapses in epileptogenesis in the adult hippocampus, we coapplied kainate and bicuculline. This procedure led to the generation of spontaneous and evoked seizures, confirming that operative GABA synapses are not required for epileptogenesis in adults (n = 3). Therefore, GABA(A) receptor antagonists are ictogenic in both adult and neonatal neurons but epileptogenic only in adults.

Discussion

Our results suggest that GABAergic synapses are critical for epileptogenesis in the neonatal, but not the adult hippocampus. Seizures must contain FOs to induce permanent alterations of the network and this requires operative GABA receptors in the developing, but not the adult brain. These observations have several implications concerning the links between maturation, GABAergic synapses, network-driven oscillations, and epileptogenesis.

GABAergic Synapses Are Not Required for Epileptogenesis in Adults

Although the present study did not focus on adult networks, our observations confirm and extend an abundant literature on the role of GABAergic synapses in the generation of γ band frequencies, FOs, and seizures. Both γ band frequencies and fast—or very fast—oscillations can be generated in vitro by carbachol, kainate, high-frequency stimulation, or zero Mg^{2+} medium (Whittington et al., 1995, 1997; Traub et al., 1996, 1998, 2005; Fisahn et al., 1998, 2004; Bracci et al., 1999; Köhling et al., 2000; Gloveli et al., 2005, Draguhn et al., 1998; Maier et al., 2003; Dzhala and Staley, 2004). Gamma oscillations require functional GABAergic synapses, whereas FOs do not (Draguhn et al., 1998; Traub et al., 2001,

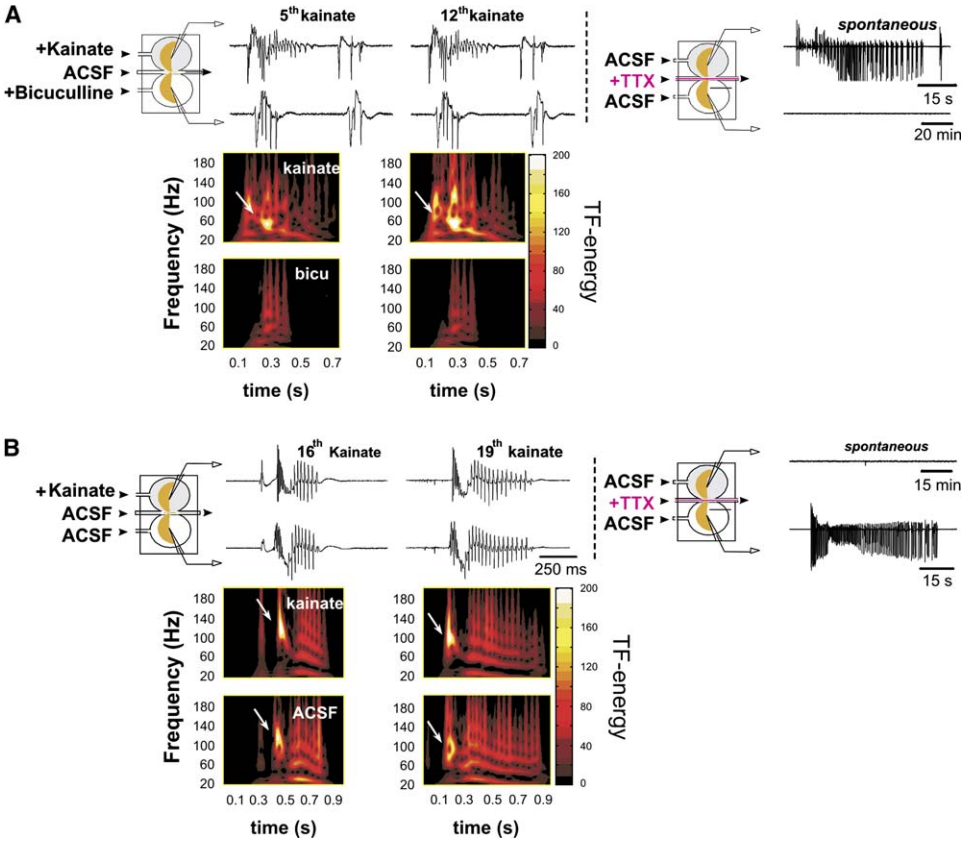

Figure 4. Causal Relation between GABA, FOs, and Formation of a Mirror Focus

(A) Field recordings: fast oscillations are indicated by white arrows. Kainate was applied to one hippocampus and ACSF + bicuculline to the other. Repeated applications of kainate failed to generate a mirror focus, as spontaneous seizures were recorded in the kainate-treated but not the bicuculline-treated hippocampus.

(B) After washout of bicuculline, the experiment was repeated while ACSF was applied to the contralateral hippocampus without bicuculline. Note that FOs were recorded only when bicuculline was not applied, suggesting that functional GABA receptors are required for their generation. A mirror focus was now established as spontaneous seizures were recorded in the contralateral hippocampus when disconnected from the kainate-treated hippocampus. As in earlier observations (see Figures 2 and 3), the kainate-treated side generated spontaneous seizures (data not shown).

2005; Bragin et al., 1999, 2002; Maier et al., 2003; Dzhala and Staley, 2004). Thus, GABA receptor antagonists block the γ components but augment the FOs that may depend on recurrent excitatory synaptic transmission (Dzhala and Staley, 2004) or nonsynaptic mechanisms such as, notably, gap junctions (Draguhn et al., 1998, Fukuda and Kosaka, 2000; Hormuzdi et al., 2001; Traub et al., 2005, Fisahn 2005). These observations suggest therefore that FOs frequencies—and GABA interneurons that are instrumental for their generation (see Steriade et al., 1998)—are not required for epileptogenesis in the adult network. Other mechanisms, including gap junctions (Traub et al., 2005), are likely to trigger the generation of FOs and the long-lasting alterations of the circuit.

Whereas the ictogenic actions of GABA receptor antagonists have been extensively studied, little is known about their epileptic actions and, in particular, whether functional GABAergic synapses are required for seizures to beget seizures. In an earlier study, we showed that brief applications of kainate lead to NMDA receptor-dependent long-lasting alterations of the network that becomes epileptic (Ben-Ari and Gho, 1988). The present results suggest that seizures generated by GABA receptor antagonists, seizures in which GABAergic synapses are fully blocked, are epileptogenic in the adult hippocampus. Following GABA(A) receptor antagonist treatment, all-or-none evoked seizures were generated by electrical stimuli that evoked only an EPSP prior to the treatment. Therefore, adult networks can generate FOs by mechanisms that do not rely on GABAergic synapses including, possibly, gap junctions, suggesting that fast oscillations can be synchronized in pyramidal neurons without the synchronizing action of GABAergic interneurons (also see Traub et al., 2005).

GABAergic Synapses and FOs Are Required for Epileptogenesis in the Neonatal Hippocampus

In neonatal slices, at approximately the end of the first postnatal week, GABA receptor antagonists generate seizures, since the shunting action of GABA is operative (Gao et al., 1998; Khalilov et al., 1999b) and since GABA inhibits a significant percentage of neurons (Tyzio et al., 2003). Seizures generated by GABA receptor antagonists in immature networks, however, do not include the FOs observed in adults, suggesting that the

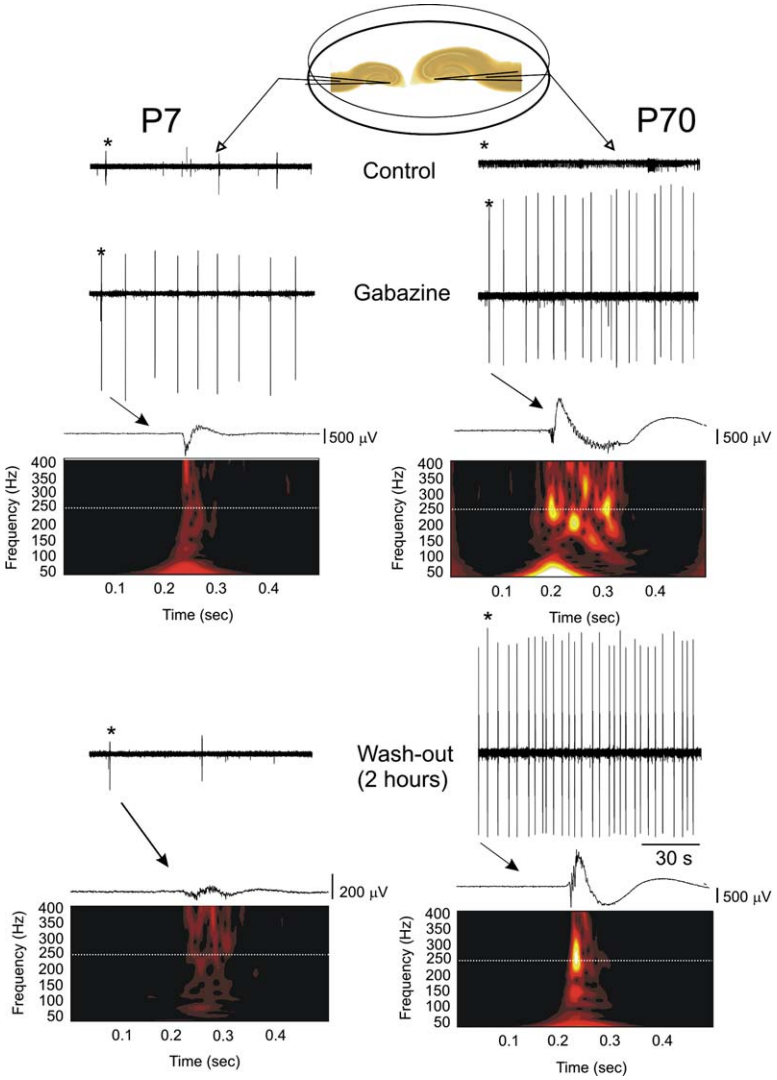

Figure 5. Epileptogenic Actions of GABA in the Developing, but Not the Adult, Hippocampal Slices

A neonatal (P7) and an adult (P30–P75) slices were placed in the same chamber, and a GABA(A) receptor antagonist (gabazine or bicuculline, 10 μM) was applied. Seizures were generated in both slices, but FOs were observed only in the adult slices. After washout of antagonists, spontaneous and evoked seizures and FOs were present in the adult, but not the neonatal, slice.

mechanisms required for the generations of FOs, including gap junctions between the axons of principal cells and/or excitatory glutamatergic synapses, may not be sufficiently mature. The seizures also neither include FOs nor lead to long-lasting consequences, suggesting that the GABAergic network is necessary both for the generation of FOs and for epileptogenesis in the immature hippocampus. Purely glutamatergic seizures cannot transform a naive network to one that spontaneously generates seizures. This is best illustrated by the experiments in which kainate and GABA receptor antagonists were applied to the same intact hippocampus: although severe seizures were generated in the treated hippocampus, both FOs and the epileptogenic actions of kainate were abolished locally, providing direct evidence of a causal link between FOs and epileptogenesis. Also, the seizures generated locally in the treated hippocampus propagated to the naive contralateral hippocampus where FOs were generated. Spontaneous seizures developed in the contralateral hippocampus, but not in the treated hippocampus, despite the coapplications of two powerful convulsive agents. These observations suggest that low-frequency events can generate FOs distally, provided that GABAergic synapses are functional. In keeping with the central role of FOs, high-frequency electrical stimuli were epileptogenic at high, but not low, frequencies. Furthermore, blocking NMDA receptors both eliminates FOs and the long-term consequences of seizures (Khalilov et al., 2003). Therefore, the density of GABAergic and glutamatergic synapses is sufficiently developed at the end of the first postnatal week to generate FOs and epileptogenic transformation of the network, but that requires the coordinated efforts of GABA and NMDA receptor-mediated synapses. In keeping with this, the hippocampus cannot generate γ and FOs at an earlier stage (P0–P2) (data not shown). Preliminary observations suggest that seizures also do not lead to long-lasting consequences at that stage (Quilichini et al., 2002). Consistent with these findings, recent studies also suggest that maturation of basket interneurons that are required for the generation of γ does not occur until several days after birth (Gozlan and Ben Ari, 2003; Ruusuvuori et al., 2004). Present observations, therefore, suggest a biphasic maturation of epileptogenic mechanisms: first, maturation of the GABA-dependent γ-generating network, at approximately the

end of the first postnatal week, and subsequently, at an as yet undefined stage, maturation of the mechanisms that generate FOs. Only at that stage do seizures beget seizures, providing interesting information about the maturation of epileptogenesis. Future experiments are however required to determine the mechanisms that underlie the formation of a mirror focus, i.e., number of spikes, frequency, or rhythmic firing of specific populations of neurons.

The Multiple Facets of Chloride Regulation and Seizures

In developing networks, as in adult networks, seizures induce an accumulation of chloride and an inhibitory to excitatory shift of GABA (Köhling et al., 2000; Cohen et al., 2002; Fujiwara-Tsukamoto et al., 2003; Isomura et al., 2003; Khalilov et al., 2003). The neuron-specific K-Cl cotransporter plays an important role in both developmental and activity-dependent control of these shifts (Rivera et al., 2004, 2005). Thus, at an early stage, seizures will produce a H to D shift (from hyperpolarization to depolarization) in the more developed neurons. This action will summate with the depolarizing action of GABA in the population of very immature neurons in which the developmentally regulated D to H shift (from depolarization to hyperpolarization) has not yet occurred. Since, in addition, the formation of a mirror focus is associated with a chronic H to D shift in GABA activity (Khalilov et al., 2003), alteration of the actions of GABA appears to play a particularly important role in both ictogenesis and epileptogenesis in the developing brain.

The observations that both GABA and NMDA receptors are required for the generation of γ and to produce the epileptogenic actions of seizures strongly suggest that the GABA/NMDA synergy is a central mechanism. In an earlier study, we showed that the excitatory action of GABA in developing neurons is sufficient to remove the voltage-dependent Mg^{2+} blockade from these channels, leading to a large calcium influx associated with neuronal plasticity (Leinekugel et al., 1997). We suggest that a vicious cycle operates in the immature hippocampus, with FO-containing seizures increasing the excitatory action of GABA synapses due to an accumulation of chloride; this removes the voltage-dependent Mg^{2+} blockade of NMDA receptors. Thus, the synergistic actions of GABA and NMDA (Leinekugel et al., 1997) will facilitate the generation of further oscillations. This suggests that FOs may be particularly powerful in triggering the H to D shift. The activation by GABA of NMDA receptors is instrumental in the shift from acute to chronic seizures, in keeping with the extensive data suggesting that NMDA receptors play a central role in physiological and pathological mechanisms underlying long-term alterations of synaptic efficacy (Ben-Ari and Gho, 1988; Malenka and Nicoll, 1993; Ben Ari and Represa, 1990). Interestingly, recurrent seizures with γ components and FOs also facilitate the synaptic efficacy of glutamatergic synapses (Velazquez and Carlen, 1999). Therefore, much like alterations of synaptic efficacy—thought to play an important role in learning and memory processes—synchronized patterns may activate NMDA receptors, leading to persistent shifts of synaptic efficacy in networks.

High-frequency oscillations are prominent at the onset of seizures in epileptic patients (Fisher et al., 1992; Worrell et al., 2004) and in several animal models of epilepsy (Bragin et al., 1999; Grenier et al., 2003), raising the possibility that they may be causally related to ictogenesis (Köhling et al., 2000; Traub et al., 2001, 2005). One important implication of the present observations is that FOs containing seizures are also directly implicated in epileptogenesis in the immature brain and lead to the production of a persistent, chronic epileptic condition. Seizures generated at an early developmental stage when a significant component of the excitatory drive is mediated by GABAergic synapses do not include FOs and will not lead to epileptogenesis. As the network matures, the density of glutamate synapses increases, whereas the contribution of GABAergic synapses to the generation of FOs and to epileptogenesis decreases. This information may be important both for understanding the deleterious consequences of seizures in newborns and for developing new therapeutic treatments for seizures in young infants. Specifically, the permissive action of excitatory GABA suggests that GABA-acting drugs may exert deleterious actions at an early developmental stage in humans.

Experimental Procedures

Hippocampal Preparation

The Interconnected Intact Hippocampal Formations (IIHFs) were prepared as described previously (Khalilov et al., 1997, 2003). In brief, neonatal (P6–P7) male Wistar rats were decapitated after hypothermic anaesthesia, and the brain was rapidly removed to oxygenated (95% O_2/5% CO_2), ice-cold oxygenated artificial cerebrospinal fluid (ACSF). Complexes including two interconnected hippocampi were isolated and transferred into a beaker of oxygenated ACSF containing: 126 mM NaCl, 3.5 mM KCl, 2.0 mM $CaCl_2$, 1.3 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM $NaH2PO_4$, and 11 mM glucose (pH, 7.4), at least 1 hr before use. IIHFs were placed into a conventional, fully submerged three-compartment chamber superfused with oxygenated physiological solution at 28–30ºC at a rate of 10–15 ml/min. The IHFs were fixed to the Sylgard bottom using entomological needles.

Slice Preparation

Slices were prepared as described previously (Ben-Ari and Gho, 1988). In brief, after sacrificing the rat (neonatal P7 and adult P45–P70) by decapitation, the brain was rapidly removed and placed in oxygenated, ice-cooled ACSF; hippocampal transverse slices (450 μm) were cut with a McIlwain tissue chopper and kept in oxygenated ACSF at room temperature, at least 1 hr before use. Individual slices were then transferred to the recording chamber where they were fully submerged and superfused with oxygenated ACSF at 30–32Cº, at a rate of 2–3 ml/min.

Electrophysiological Recordings

All the recordings were made in the area CA3. Whole-cell recordings were performed with the use of a patch-clamp technique in voltage-clamp or current-clamp configurations using Axopatch 200B (Axon Instruments, USA). The whole-cell patch pipettes had a resistance of 8–10 MΩ when filled with solution containing: 135 mM K-gluconate, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Mg-ATP, 10 mM ethylene glycol-bis (B-aminoethyl ether)-N,N,N,N tetraacetic acid (EGTA) 1 and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (pH 7.25; osmolarity, 270 mOsm; membrane potential values were corrected for a liquid potential of +13 mV. Cell-attached recordings were performed using glass micropipettes filled with ACSF. Cells were identified by adding biocytin (0.4%) to the pipette solution for morphological analysis.

Extracellular field potentials were recorded conventionally using glass micropipettes (1.2 mm O.D. × 0.94 mm I.D., GC120TF-10;

Clark Electromedical Instruments) filled with ACSF (1–5 MΩ) and DAM-80 amplifiers (WPI, GB; low filter: 0.1 Hz; high filter: 3 KHz). Electrical stimulations (10–20V, 40 μs) were provided by a bipolar electrode placed in the CA3 hippocampal region. Synaptic responses were acquired into the memory of a personal computer using an analog-to-digital converter (Digidata 1200; Axon Instruments, USA). Axoscope 8.0, Clampfit 8.0 (Axon Instruments, USA) and Origin 7.0 (Microcal Software, USA) programs were used for the acquisition and analysis of the synaptic activities. Group measures are expressed as the mean ± SEM; error bars also indicate ±SEM. Statistical significance of differences was assessed with the Student's t test; the level of significance was set at $p < 0.05$. Drugs used were purchased from Sigma (tetrodotoxin), Tocris (bicuculline, gabazine, CNQX, and D-APV), and Molecular Probes (biocytin).

Data Analysis

Because neither latency nor frequency of oscillatory bursts was known *a priori*, a time-frequency (TF) representation that preserves both types of information was chosen (Thakor and Tong, 2004). The main advantage of this approach is that the duration of the window of analysis depends on the frequency band: the higher the central frequency, the shorter the window duration and the wider the frequency band. In our work, the signal was convolved with complex Morlet's wavelets $w(t, f_0)$ having a Gaussian shape both in the time domain ($SD = s_t$) and in the frequency domain ($SD = s_f$) around its central frequency f_0: $w(t, f_0) = A.\exp(t^2/2s_t^2) \times \exp(2ipf_0t)$, with $s_f = 1/2ps_t$. The wavelet family that we used was defined by $f_0/s_f = 5$, with f_0 sampled logarithmically from 10 to 200 Hz. Here, 50 frequency steps were used. The time-varying energy $E(t, f_0)$ of the signal in a frequency band around f_0 is the squared norm of the result of the convolution of a complex wavelet $w(t, f_0)$ with the signal $s(t)$: $E(t, f_0) = |w(t, f_0) \times s(t)|^2$. As a criterion of the significance for the TF representations, we required that the TF peak energy exceed the mean +5 SD of a baseline taken far away from ictal events. Thus, by using the background signal in the same way at each frequency, the method allowed us to statistically control the levels of oscillatory activity from those that one would expect by chance and to fairly compare oscillatory episodes across frequencies. To detect oscillatory events, we designed an algorithm to identify the periods within the signal that exhibits high-power oscillatory activity at a particular frequency, lasting a few cycles. We defined oscillatory events by detecting local maxima in the normalized TF representations with a duration longer than K cycles at a particular frequency, during which the power exceeded N standard deviations of baseline. We have adjusted the thresholds (K, N) to be relatively insensitive to sharp transients, as found in epileptic recordings. Concerning γ oscillations at the onset of seizures, the duration threshold was set to 5 cycles (i.e., $5/f$ sec), and the amplitude threshold was set to 3 SDs. This method allowed us to compare the incidences of oscillations between different conditions.

Supplemental Data

Supplemental Data include three figures and are available online at http://www.neuron.org/cgi/content/full/48/5/787/DC1/.

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