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Maturation of kainate-induced epileptiform activities in interconnected intact neonatal limbic structures *in vitro*

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Abstract

In vivo studies suggest that ontogenesis of limbic seizures is determined by the development of the limbic circuit. We have now used the newly-developed *in vitro* intact interconnected neonatal rat limbic structures preparation to determine the developmental profile of kainate-induced epileptiform activity in the hippocampus and its propagation to other limbic structures. We report gradual alterations in the effects of kainate during the first postnatal week on an almost daily basis; from no epileptiform activity at birth, through interictal seizures around postnatal day (P) 2 and ictal seizures by the end of the first week. The developmental profile of kainate-induced hippocampal seizures is paralleled by the expression of postsynaptic kainate receptor-mediated currents in CA3 pyramidal cells. Intralimbic propagation of the hippocampal seizures is also age-dependent: whereas seizures readily propagate to the septum and to the contralateral hippocampus via the commissures on P2, propagation to the entorhinal cortex only takes place from P4 onwards. Finally, repeated brief applications of kainate to the hippocampus induce recurrent spontaneous glutamatergic ictal and interictal discharges which persist for several hours after the kainate is washed away and which replace the physiological pattern of network activity. Paroxysmal activities are thus generated by kainate in the hippocampus at an early developmental stage and are initially restricted to this structure. Before the end of the first week of postnatal life, kainate generates the epileptiform activities that may perturb activity-dependent mechanisms that modulate neuronal development. Although at this stage neurons are relatively resistant to the pathological effects of kainate, the epileptiform activities that it generates will perturb activity-dependent mechanisms that modulate neuronal development.

Introduction

The incidence of seizures in humans is highest in early postnatal life, suggesting that the immature brain is more seizure-prone than the adult (Moshe & Cornblath, 1993; Hauser, 1995; Holmes, 1997; Holmes & Ben-Ari, 1998). Studies using animal models have consistently shown that immature neurons are far more resistant than those of adults to the pathological consequences of seizures yet recurrent neonatal seizures are often associated with long-term neurological disorders (ibid and Nitecka *et al*., 1984; Ben-Ari, 1985; Holmes *et al*., 1998). This suggests that seizures may produce their adverse effects not only by neuronal cell loss but also by altering processes that are essential for activity-dependent network formation (see Constantine-Paton *et al*., 1990; Goodman & Shatz, 1993; LoTurco *et al*., 1995; Komuro & Rakic, 1996; Ben-Ari *et al*., 1997). Understanding these mechanisms is important as it is often considered that infantile seizures do not require aggressive treatment because of the lack of neuronal loss and the adverse effects in children of therapeutic agents (Holmes, 1997; Mitaki *et al*., 1994; Holmes & Ben-Ari, 1998).

Developmental studies on kainate-induced epileptic activity in the rat limbic system allowed important insights into the maturation of the limbic seizures syndrome. In adult rats, parenteral or intraventricular injection of kainate induces the following sequence of correlated behavioural and electrographic abnormalities (reviewed in Ben-Ari, 1985): (i) first, localized paroxysmal discharge in the hippocampus; (ii) next, individual recurrent limbic seizures extended to other limbic structures; and (iii) finally, full status epilepticus with generalization of paroxysmal activity to nonlimbic structures. This sequence of events has also been described using functional mapping with 2-deoxyglucose (2-DG) autoradiography (Tremblay *et al*., 1984; White & Price, 1993) and fos immunostaining (Popovici *et al*., 1990). Repeated limbic seizures also lead to a pattern of brain damage that is reminiscent of the damage that occurs in human temporal lobe epilepsy (TLE) suggesting that the kainate syndrome provides a useful animal model of TLE (Nadler, 1979, 1981; Ben-Ari, 1985). In contrast, parenteral injections of kainate in immature rats induce tonic or tonic–clonic convulsions, limbic motor seizures being first observed from the end of the third week of postnatal life (Tremblay *et al*., 1984; Cherubini *et al*., 1983; Albala *et al*., 1984; Stafstrom *et al*., 1992). Functional mapping using 2-DG labelling suggest that kainate-induced effects are at birth exclusively restricted to the CA3 region of both hippocampi and the lateral septum (Tremblay *et al*., 1984). These *in vivo* data therefore suggest that although the hippocampus has the lowest threshold for the epileptogenic effects of kainate, the propagation of epileptiform activities from the hippocampus to other limbic structures has an important developmental profile reflecting a functional maturation of limbic circuitry. The difficulty of recording during early developmental stages in neonatal brains has, however, precluded a

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determination of the generation and propagation of epileptiform activities in the immature limbic system.

We have now used for the first time a preparation that has been recently developed in this laboratory in order to re-examine the developmental profile of the epileptogenic effects of kainate using multiple field and patch clamp recordings. This preparation allows recordings to be made from intact interconnected limbic structures *in vitro* and the use of all the techniques used in conventional slices (Khalilov *et al*., 1997b). We also used another technological approach that enables us to place the two hippocampi and their connecting commissures in three different compartments (Khazipov *et al*., 1999). This enables us to test the propagation of epileptiform activities from one hippocampus to the other. Three issues were addressed in the present study: (i) what is the developmental profile of seizure activity and how does this correlate with the maturation of kainate receptors in CA3 pyramidal neurons?; (ii) what are the properties of epileptiform activity and at what developmental stage does it propagate to the contralateral hippocampus and to other limbic structures?; (iii) do seizures alter the pattern of activity present in the immature hippocampus and most notably the characteristic giant depolarising potentials (GDPs) that provide at this early stage most of the neuronal activity (Ben-Ari *et al*., 1989; Khazipov *et al*., 1997; Leinekugel *et al*., 1997, 1998). We report that kainate generates powerful paroxysmal activities before the end of the first week of postnatal life and profoundly alters the GDPs. Initially restricted to the hippocampus, seizures propagate at P2 to the other hippocampus and, in an age-dependent manner, also to other limbic structures. Kainate may have deleterious actions in the developing hippocampus by altering activity-dependent mechanisms that are essential for the formation of the circuit.

Materials and methods

Preparation and maintenance of hippocampal slices and IHFs

The intact hippocampal formations (IHFs) were prepared as described previously (Khalilov *et al*., 1997b). In brief, neonatal [postnatal days P0–P8] male Wistar rats were decapitated after hypothermic anaesthesia and the brain was rapidly removed to oxygenated (95% O_2 : 5% CO_2) ice-cold physiological solution. Complexes including two hippocampi, septum and entorhinal cortex or single hippocampi were isolated and transferred into a beaker containing oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 126; KCl, 3.5; $CaCl_2$, 2.0; $MgCl_2$, 2.0; $NaHCO_3$, 25; $NaH2PO_4$, 1.2; and glucose, 11 (pH 7.4), and kept there at least 1 h before use. For experiments on slices, hippocampal transverse slices (500 μM) were cut from IHFs using a tissue chopper and kept in physiological solution at room temperature as described previously (Ben-Ari *et al*., 1989). The IHFs were placed in a conventional fully-submerged chamber superfused with oxygenated physiological solution at 30–32 °C at a rate of 10–15 mL/min. The two-compartment recording chamber has been described previously (Khalilov *et al*., 1997b). The three-compartment chamber has two principal differences from the two-compartment chamber: (i) the third compartment is introduced between the two hippocampal chambers with a groove to lodge and perfuse separately the interhippocampal commissure; (ii) the common outlet is used for all three compartments, preventing leakage between the chambers (see below, Fig. 9). Recently we have developed a novel type of the three-compartment chamber that is based on the use of latex membranes to separate the compartments (Khazipov *et al*., 1999).

Electrophysiological recordings

All the recordings were made in the hippocampal areas CA3/CA1 of IHFs and in transverse hippocampal slices. Whole-cell recordings were performed with the use of the patch-clamp technique in voltage-clamp or current-clamp configurations using Axopatch 200D (Axon Instruments, USA) and EPC-9 (HEKA Electronics, Germany) patch-clamp amplifiers. Patch electrodes were made from borosilicate glass capillaries (outside diameter 1.5 mm, inside diameter 0.86 mm; type GC150F-15, Clark Electromedical Instruments) and had a resistance of 5–10 MΩ when filled with solutions containing (in mM): (i) K-gluconate, 135; $CaCl_2$, 0.1; Mg-ATP, 2; ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 1; and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 10, pH 7.25, osmolarity 280 mosm; or (ii) CsCl, 140; $CaCl_2$, 1; EGTA, 10; HEPES, 10; MgATP, 2, pH 7.25, osmolarity 270–280 mosm. Cells were identified under infra-red microscopy or by adding lucifer yellow (0.5%) and/or biocytin (0.4%) to the pipette solution for morphological analysis (Khalilov *et al*., 1997a). Extracellular field potentials were recorded using glass microelectrodes (outside diameter, 1.2 mm; inside diameter, 0.94 mm; GC120TF-10, Clark Electromedical Instruments) with resistances of 10–20 MΩ when filled with ACSF and using a DAM-80C AC differential amplifier (World Precision Instruments, USA) set at 0.1 to 1–3-kHz band-pass.

Synaptic current responses were acquired on a DAT tape recorder (Biologic, France) and into the memory of an 80486 personal computer using a Labmaster interface (USA). Data were acquired and analysed using Acquis Software (Gerard Sadoc, France), pClamp5.1 (Axon Instruments, USA), Origin 5.0 (Microcal Software, USA) and Axotape (Axon Instruments, USA).

Fluorescence measurements of $Ca^{2+}{}_i$

Fluorescence measurements were performed as described (Khalilov *et al*., 1997a). In brief, neurons were loaded with the Ca^{2+}-sensitive dye Fluo-3 in the esterified form (Fluo-3 AM, 3.3 μM applied focally from a micropipette for 5–30 min with 0.3–1 s, 0.2 Hz pressure pulses). Fluorescence was measured using a confocal laser scanning microscope (MRC BIORAD 600) equipped with an argon–krypton laser and photomultiplier. Excitation was delivered at 488 nm and emission intensity was measured at a wavelength > 500 nm. Images were acquired every 0.5–4 s using program SOM (BIORAD, USA) and analysed off-line with the program Fluo (IMSTAR, France). All results were expressed as ΔF/Fmin, where F is the fluorescence from the defined portion of the image corresponding to the cell(s) under investigation and Fmin is the mean baseline fluorescence in the selected area(s) from at least five consecutive images.

Ionic measurements

Ionselective microelectrodes were made from double-barrelled borosilicate glass (2GC150FS, Clark Electromedical, Pangbourne, Reading, UK) when the filamented barrel was used for field potential recording. The nonfilamented barrel was exposed to vapor of dimethyl-trimethyl-silylamine (TMSDMA, Fluka, Buchs, Switzerland). After baking at 200 °C, the pipettes were bevelled dry and the nonfilamented barrel was backfilled (with the use of pressure) with the filling solution. A short column of the ion sensor was taken into the tip of the silanized barrel by using slight suction. Ion sensors for K^+ and Ca^{2+} were purchased from Fluka (Fluka 60398 and Fluka 21196, respectively). Composition of the filling solutions in the nonfilamented barrels were (in mM): NaCl, 150; KCl, 3 for K^+-selective electrodes; and NaCl, 100;

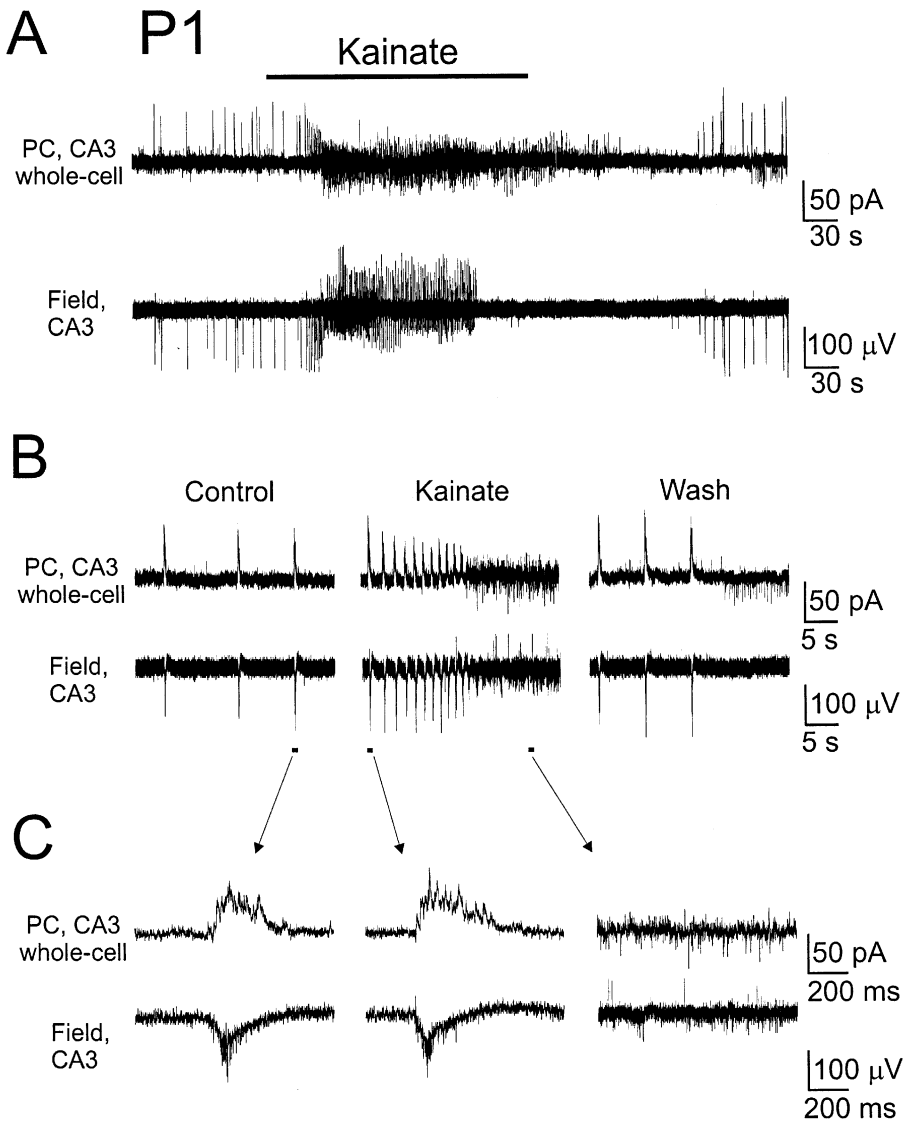

FIG. 1. Simultaneous field (Field) and whole-cell recordings from CA3 pyramidal cells in voltage-clamp mode (PC); holding potential, –50 mV. Effects of kainate on P1 intact hippocampus. (A) Simultaneous recordings of the CA3 pyramidal cell (upper traces) and extracellular field (lower traces) in the pyramidal layer of the CA3 subfield in the new-born (P1) rat hippocampus. The CA3 pyramidal cell was recorded in whole-cell voltage-clamp mode at –50 mV so that GABAergic responses are outwardly and glutamatergic are inwardly directed. (B) Parts of traces from A on an expanded time scale. Note that, following the kainate application, the frequency of spontaneous giant depolarising potentials (GDPs) is initially increased and this is followed by a phase of desynchronization of the neuronal activity. (C) GDPs in control (left), at the initial phase of kainate action (middle) and the phase of desynchronization of the neuronal activity (right). Note the increased frequency of spontaneous IPSCs and EPSCs in whole-cell recordings associated with increased frequency of unit activity in field recordings during the desynchronization phase. No collective activity is observed either in field or in whole-cell recordings.

HEPES, 1; $CaCl_2$, 1; and NaOH, 0.5 in Ca^{2+}-selective electrodes. The resistance of the electrodes was 5–15 GΩ. The K^+ electrodes had a slope of ≈57 mV per decade change. The Ca^{2+} electrodes had a slope of 26–28 mV per decade.

Statistics

Unless stated otherwise, data are expressed as means ± SEM. Statistical significance of differences between means was assessed with Student's *t*-test, with the aid of statistical software StatView SE$^+$ Graphics^{TM}. The level of significance was set at $P < 0.05$.

Drugs

Drugs were purchased from Sigma (St.Louis, MO, USA), molecular probes (Fluo3 and fluo3-AM) from Tocris Neuramin. GYKI 56355 was kindly provided by D. Leander (Eli Lilly, Indianapolis).

Results

Hippocampal seizures build up during the first postnatal week

To determine the developmental profile of the epileptogenic actions of kainate, simultaneous extracellular field recordings in the stratum pyramidale of CA3 and whole cell recordings from CA3 pyramidal neurons or interneurons were performed in the IHF preparation (Khalilov *et al.*, 1997b).

In the P0–P1 IHFs, the pattern of activity in the hippocampal network in control conditions was characterized by spontaneous GDPs. GDPs were simultaneously recorded in whole-cell recordings from CA3 pyramidal cells and extracellular recordings from the CA3 pyramidal layer (Fig. 1) and occurred at a frequency of 4.6 ± 1.3 per minute ($n = 5$). The typical response to kainate (250 nM) was biphasic. During the first phase, the frequency of GDPs transiently increased up to 1.0 ± 0.2 per second ($n = 5$). This was followed by a phase of desynchronization during which GDPs were suppressed (Fig. 1B and C). During this phase, the frequency of spontaneous γ-aminobutyric acid (GABA)-inhibitory post-synaptic currents (IPSCs) and the excitatory post-synaptic currents (EPSCs) as well as unit activity in field recordings increased. However, there were no population spikes in field and polysynaptic events in whole-cell recordings. Moreover, an even higher concentration of kainate (1 μM) still failed to induce epileptiform activity (not shown). Thus, in the P0–P1 IHFs, no paroxysmal activity was observed in response to kainate.

At P2–P3, the response to kainate (250 nM) in 48% of cases ($n = 10$ of 21) was similar to that observed in the earlier ages, i.e. increased frequency of GDPs followed by desynchronization of neuronal

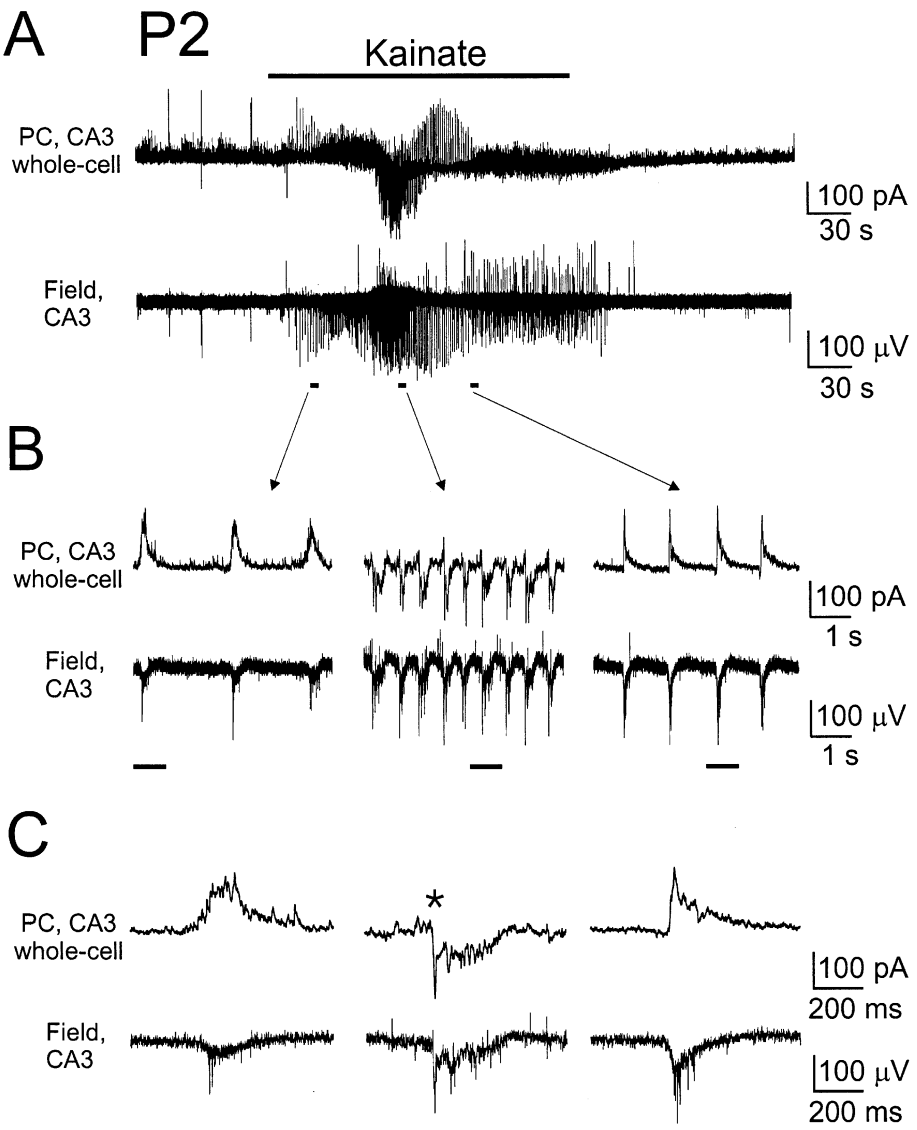

FIG. 2. In P2 intact hippocampus, kainate induces interictal-like activities. (A) Simultaneous recordings of the CA3 pyramidal cell and extracellular field in the 2-day-old rat (P2) hippocampus. A pyramidal cell in the CA3 subfield was recorded in whole-cell voltage-clamp mode at –50 mV so that GABAergic responses are outwardly and glutamatergic responses are inwardly directed. (B) Traces from A on an expanded time scale: phases of the increased frequency of GDPs (left), of interictal-like activities with a dominance of the glutamate (middle) and GABA-A (right) -receptor-mediated postsynaptic conductances. (C) Single population events from panel B on an expanded time scale. Note in the middle trace that the interictal event starts with a collective population spike in the field recording (*) associated with an EPSC in the pyramidal cell, and is followed by afterdischarge with a dominance of the glutamate receptor-mediated (inwardly directed) conductance in the pyramidal cell.

activity. In 52% of cases, a phase of the increased frequency of GDPs was followed by a brief sequence of interictal bursts (Fig. 2, $n = 11$ of 21). Interictal bursts were characterized by collective spikes in field recordings associated with polysynaptic events in whole-cell recordings (Fig. 2B and C, middle traces). Whole-cell recordings with a low-chloride pipette solution that allows separation of GABA-A and glutamate receptor-mediated currents (Khazipov *et al.*, 1997) revealed that the interictal events are associated with glutamate receptor-mediated currents in CA3 pyramidal neurons (Fig. 2B and C). In one case out of 21, ictal-like activity with a characteristic phase of tonic network oscillations followed by clonic afterdischarges was observed.

In P4–P7 IHFs, kainate (250 nM) generated tonic–clonic ictal episodes in 56% of cases ($n = 23$ of 41, Fig. 3). Activity of CA3 pyramidal cells was synchronized with population activity through all phases of the kainate-induced hippocampal seizure activity. As shown in Fig. 3, kainate-induced ictal episodes developed through four principal phases. Epileptiform activity started with several large-amplitude (0.5–2 mV) collective discharges. After these large-amplitude discharges the network fell into the tonic phase, characterized by rhythmic oscillations of 6–20 Hz that had a tendency to decrease at the end of the tonic phase. The tonic phase was followed by a series of clonic discharges (Fig. 3). Ictal-like discharge was terminated by postictal depression during which the network activity was suppressed. Thus, epileptiform activity induced by kainate in the neonatal hippocampus has a sequence of phases typical of the ictal episodes described *in vivo* and *in vitro* (Matsumoto & Ajmone-Marsan, 1964; Bragin *et al.*, 1997) and activity of CA3 pyramidal cells is synchronized with the population activity through all phases of the paroxysmal activity.

The first postnatal week is thus a critical period for epileptogenesis in the hippocampus (Fig. 4). During this narrow temporal window, kainate-induced seizures are building up day after day, starting from virtually no responses at birth, through the interictal to full-blown ictal seizures at the end of the first week.

Epileptogenesis is paralleled by postsynaptic response to kainate

In adult hippocampus, epileptiform activity induced by kainate is generated by the activation of kainate receptors present on CA3 pyramidal neurons (see Discussion). Therefore in the next series of experiments we asked whether the developmental profile of the epileptogenic action of kainate might be explained by maturation of the sensitivity of CA3 pyramidal cells to kainate during this

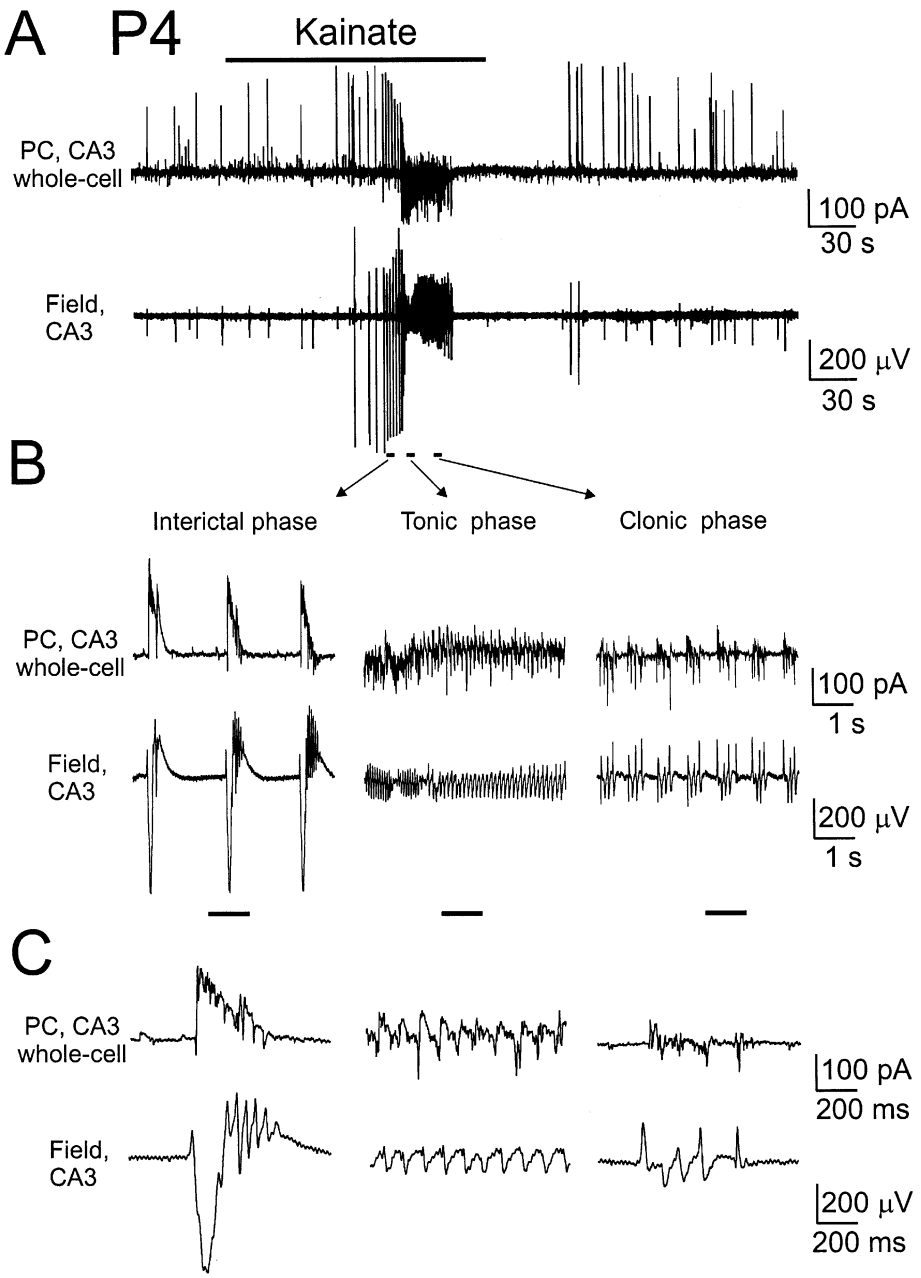

FIG. 3. In P4 intact hippocampus, kainate induces ictal-like tonic–clonic discharges. (A) Bath-applied kainate (250 nM) generates synchronous tonic–clonic ictal-like activity in the field recordings in the CA3 *stratum radiatum* and whole-cell recordings from a CA3 pyramidal cell (–60 mV). (B and C) Phases of the ictal episode on an expanded time scale.

period. To determine the developmental profile of kainate receptor-mediated currents in CA3 pyramidal neurons, kainate was applied in the presence of tetrodotoxin (TTX) (1 μM) to block sodium action potentials. As shown in Fig. 5A, in P0–P1 slices, kainate (300 nM) either did not generate a detectable current ($n = 9$) or induced an inward current of 11 ± 5 pA ($n = 4$). Starting from P2, kainate (250 nM) invariably generated in CA3 pyramidal neurons inward currents that progressively increased with age (Fig. 5A and B). Kainate-induced currents persisted in the presence of a cocktail of antagonists including, in addition to TTX (1 μM), 2-amino-5-phosphonopentanoic acid (APV; 50 μM), bicuculline (10 μM) and GYKI 56355 (30 μM) to block sodium action potentials, NMDA, GABA-A and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, respectively, and were antagonized by the nonselective AMPA/kainate receptors antagonist 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) (10–20 μM, Fig. 5A), suggesting that they are mediated by high-affinity kainate receptors. Thus, during the first postnatal week, there is a progressive expression of the postsynaptic responses to kainate that is closely paralleled by the developmental profile of the kainate-induced seizure activity.

Pharmacology of the neonatal hippocampal seizures

In adults, recurrent collaterals interconnecting pyramidal cells play the principal role in synchronization of the paroxysmal discharges (Rutecki *et al.*, 1985; Miles & Wong, 1986; Traub *et al.*, 1987). Therefore in the next series of experiments we investigated the role of the synaptic mechanisms in the generation of the paroxysmal discharges in the neonatal hippocampus. Blockade of synaptic transmission by TTX (1 μM) completely prevented generation of the paroxysmal discharges by kainate (300 nM; $n = 2$). Similarly, increased concentration of divalent cations in the perfusing solution (6 mM Mg^{2+} and 4 mM Ca^{2+}), known to suppress the network-driven polysynaptic activity (Berry & Penthreath, 1976), also prevented the generation of epileptiform activities ($n = 3$). As synaptic transmission in the recurrent collaterals is mediated by AMPA and NMDA

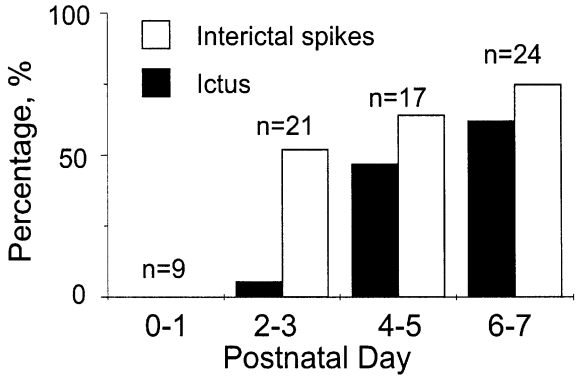

FIG. 4. Developmental profile of the epileptogenic effect of kainate during the first postnatal week. Dependence of the epileptogenic action of kainate on age. White columns show the percentage of cases in which kainate induced interictal activities and black columns correspond to the cases when ictal episodes were observed. Cases with ictal-like activity are also included in the group of interictal events; *n*, number of hippocampi in each age group.

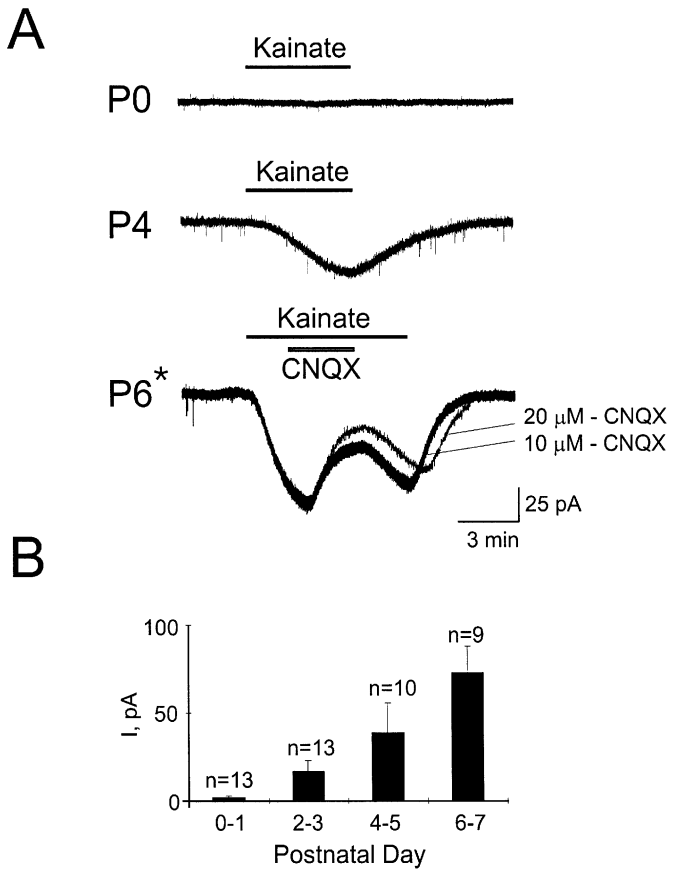

FIG. 5. Progressive expression of the postsynaptic responses to kainate in CA3 pyramidal cells during the first postnatal week. (A) Postsynaptic currents induced in CA3 pyramidal cells by bath-application of kainate (300 nM) to hippocampal slices at three different ages (postnatal days P0, P2 and P6). Kainate was applied in the presence of TTX (1 μM) to prevent the activation of the network. At P6, kainate (300 nM) induced an inward current in presence of cocktail of antagonists including TTX (1 μM), bicuculline (10 μM), APV (50 μM) and GYKI 53655 (30 μM). This current was antagonized in a dose-dependent manner by CNQX (10 and 20 μM). Whole-cell recordings with K-gluconate solution at a holding potential of –60 mV. (B) Diagram of amplitude of the kainate-induced currents at different ages; *n*, number of cells.

receptors, we further investigated the effect of these receptors antagonists. The selective AMPA receptor antagonist GYKI 53655 (30–50 μM) strongly depressed the paroxysmal discharges generated by kainate (Fig. 6, $n=4$). The remaining network activity was fully blocked by further addition of APV (50 μM, $n=4$) suggesting that the synchronization of neuronal activity during epileptiform activity is mediated by the activation of AMPA and NMDA receptors but not kainate receptors. In the presence of GYKI and APV, kainate increased multiple unit activity (Fig. 6) probably reflecting the asynchronous excitation of the pyramidal cells. Therefore, AMPA and NMDA receptors, probably located on the recurrent collateral synapses between CA3 pyramidal cells, play a pivotal role in the generation of the kainate-induced seizures in the neonatal hippocampus.

Ionic changes during epileptiform activity

The epileptiform activities in adult cortical structures are associated with large alterations in the ionic gradients (Heinemann *et al.*, 1977). Ion-selective microelectrodes were therefore used to study $[K^+]_o$ and $[Ca^{2+}]_o$ alterations in neonatal IHFs (Fig. 7). In P0–P1 IHFs, kainate produced a smooth increase of $[K^+]_o$ in the stratum radiatum to 5.0 ± 0.5 mM (from the baseline activity with 3.5 mM; $n=4$) although no epileptiform activity was observed. In older age groups, kainate-induced ictal episodes were associated with an initial rise of $[K^+]_o$ to 6.5 ± 1.0 mM ($n=16$) during the interictal activity that preceded the ictal phase. This steadily increased to reach a peak during the tonic phase of the ictal period up to 12.0 ± 1.0 mM ($n=11$) and 12.5 ± 1.0 mM ($n=5$) in P4–P5 and P7–P8 IHFs, respectively. Interestingly, in slices cut from the hippocampi of P6–P8 rats, kainate induced only interictal-like activity associated with rise of $[K^+]_o$ in the stratum radiatum to 5.0 ± 1.0 mM ($n=6$), possibly reflecting the importance of the increased connectivity in immature circuits where the density of synapses is low.

Ca^{2+}-sensitive microelectrodes revealed that $[Ca^{2+}]_o$ decreased to $73 \pm 3\%$ ($n=4$, P5) of the baseline level during the ictal period. Ictal activity in IHFs is associated with a significant shrinkage of the extracellular space, as shown by powerful accumulation of the extracellular marker TMA (tetramethylammonium) (by $\times 1.5$–2, not shown). Taking into account this shrinkage, the drop of $[Ca^{2+}]_o$ was estimated as equivalent to about 1.1 mM, because in standard bicarbonate buffer only about three-quarters of the total concentration of Ca^{2+} (2 mM) is available (Heinemann *et al.*, 1977).

Reduction of $[Ca^{2+}]_o$ is probably due to an influx of Ca^{2+} during the sustained depolarization of neurons during the epileptiform discharges. To evaluate the changes in $[Ca^{2+}]_i$, neurons were filled with the Ca^{2+}-sensitive permeable fluorescent dye Fluo-3AM (Leinekugel *et al.*, 1995). Confocal microscopy and simultaneous field recordings showed that $[Ca^{2+}]_i$ fluorescence increased during kainate-induced epileptiform activity by $42 \pm 12\%$ (Fig. 7B, $n=6$). Epileptiform activities are therefore associated with a parallel reduction of $[Ca^{2+}]_o$ and increase of $[Ca^{2+}]_i$.

Thus, hippocampal seizures in neonates are associated with an increase in the extracellular concentration of potassium ions and increase and decrease in the intra- and extracellular $[Ca^{2+}]$, respectively. Whilst accumulation of the extracellular potassium might be an important factor in the maintenance of the epileptiform activity, increase in the intracellular calcium can mediate, via the intracellular cascades, the long-term effects of the epileptic activity (see below).

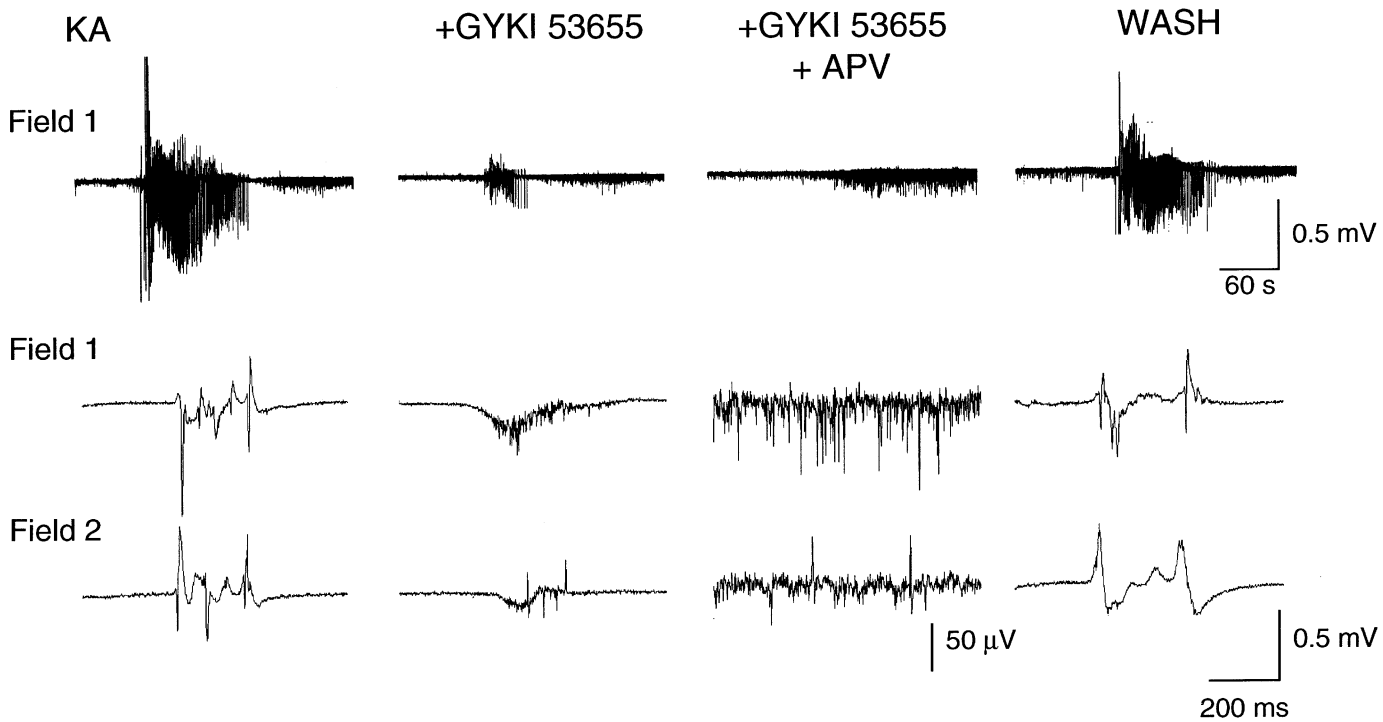

FIG. 6. Generation of kainate-induced epileptiform activity requires AMPA and NMDA receptors. Application of kainate (300 nM) induces ictal episodes in the P6 IHF (upper left trace); the epileptiform discharges during the clonic phase recorded by two electrodes located in the septal (Field 1) and temporal (Field 2) parts of the hippocampus are shown below on an expanded time scale. Selective AMPA receptor antagonist GYKI 53655 (50 μM) reduced the network discharges; the remaining synchronized events were completely blocked by the addition of APV (50 μM). In the presence of GYKI 53655 and APV, kainate generated small amplitude asynchronous spikes. Partial recovery was observed after 30 min of wash (right traces).

Developmental profile of the intralimbic propagation of the hippocampal seizure

To investigate the intralimbic propagation of the hippocampal seizures, we extended the IHF preparation to include other limbic structures: septum and entorhinal cortex (Fig. 8). At P2, kainate (300 nM) generated series of interictal discharges in the hippocampi which propagated to the septum with a latency of 54 ± 11 ms ($n = 6$). After cutting the hippocampo-septal connections, epileptiform discharges were still generated in the hippocampus but not in the septum ($n = 5$). In the P2 preparation that included hippocampus and the entorhinal cortex, hippocampal seizures did not propagate to the entorhinal cortex (Fig. 8A; $n = 4$).

At P6, kainate-induced hippocampal seizures propagated both to septum (Fig. 8B) and to entorhinal cortex (Fig. 8C). The hippocampal discharges preceded the septal ones (latency 54 ± 15 ms, $n = 4$) and cutting the septo-hippocampal connections abolished septal but not hippocampal activity, as at younger ages (Fig. 8B). However, in contrast to P2, small-amplitude epileptiform activity was now observed in field recordings from the entorhinal cortex ($n = 6$; Fig. 8C, left traces). In whole-cell recordings of layers 5–6 entorhinal cortex neurons, kainate evoked epileptiform activity synchronous with hippocampal activity in four cells and no effects in seven others (Fig. 8C). Epileptiform activity in the entorhinal cortex was abolished by cutting the hippocampo-entorhinal connections, suggesting a hippocampal origin for the activity in the entorhinal cortex (Fig. 8C). Therefore, in keeping with *in vivo* data, the hippocampus is clearly the primary site of action of kainate for generating paroxysmal activities in the developing limbic system, and propagation of the hippocampal seizure to other limbic structures is age-dependent.

Propagation to the contralateral hippocampus

We further took advantage of the *in vitro* preparation to investigate in detail the propagation of the hippocampal seizures to the contralateral hippocampus via the commissural connections. For this purpose we used a recently-developed three-compartment chamber which allows separate perfusion of the two hippocampi and the interconnecting commissural fibres (Fig. 9; see also Khazipov *et al.*, 1999). Seizures induced by application of kainate to one hippocampus propagated to the contralateral side leading to synchronized activities in both hippocampi starting from P2 ($n = 3$), suggesting an early maturation of the commissural connections (Fig. 9A). The contralateral propagation of hippocampal seizures occurred in three distinct phases (Fig. 9B): (i) the epileptiform activity was initially restricted to the ipsilateral side (exposed to kainate); (ii) in the second phase, the ipsilateral side was driving the activity in both hippocampi; (iii) in the third phase, the rebound discharges could originate in the contralateral side and backpropagate to the ipsilateral side. Contralateral propagation of the hippocampal seizure was efficiently blocked by cutting the commissural connections (Fig. 9A) or application of TTX in the commissural chamber (Fig. 9B, $n = 2$).

Long-term effects of kainate

To determine if epileptiform activities produce long-lasting alterations of hippocampal discharge, repeated applications of kainate were made. As shown in Fig. 10A, this progressively increased the duration and amplitude of ictal episodes and persistently altered the pattern of activity of hippocampal neuronal networks. Thus, the characteristic network-driven GDPs were persistently replaced after three to eight applications of kainate by spontaneous interictal and ictal activities ($n = 10$; Fig. 10B). Spontaneous ictal activities (duration 61 ± 9 s;

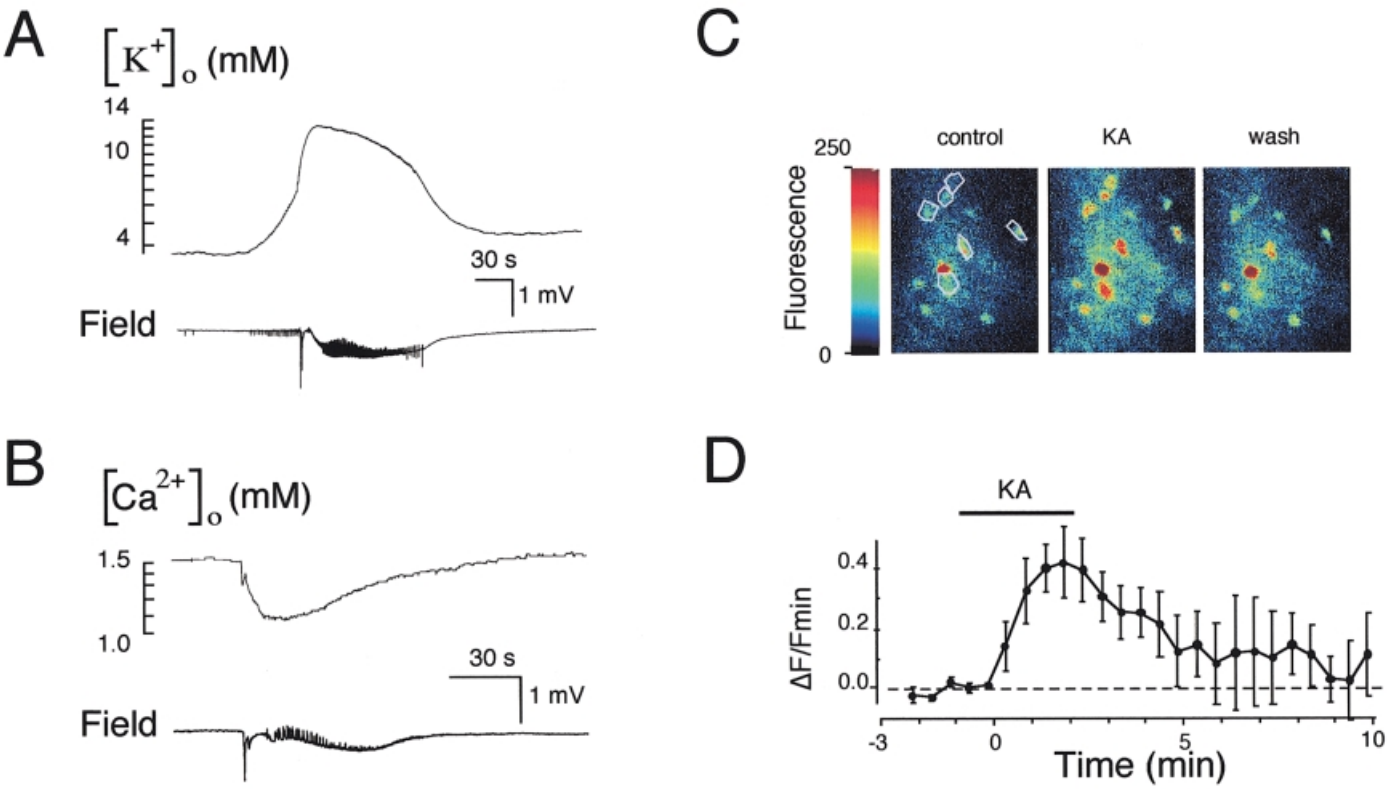

FIG. 7. Ionic changes during kainate-induced epileptiform activity. (A and B) Changes in the extracellular K^+ (A) and Ca^{2+} (B) concentration during kainate-induced ictal discharges measured by K^+- and Ca^{2+}-selective electrodes, respectively; on the lower traces, simultaneous extracellular recordings in the IHF preparation were performed in DC mode. (C) Confocal images of a group of CA3 pyramidal cells labelled with the local application of the permeable fluorescent Ca^{2+}-sensitive dye Fluo-3AM in control, after bath application of kainic acid (KA) and after 15 min wash. In the control panel, the frames outline the cell bodies in which the fluorescence was monitored. Note the reversible increase of Ca^{2+}-fluorescence after application of kainate. (D) Effect of kainate on the average intracellular Ca^{2+}-fluorescent signal (ΔF/Fmin) in six experiments.

$n = 7$) occurred repeatedly with 3–30 min intervals (15.2 ± 3.0 min; $n = 7$) and persisted for several hours (3.5 ± 0.7 h; $n = 4$). Similarly to the ictal episodes evoked by kainate, spontaneous epileptiform activities are network-driven glutamatergic events that were abolished by combined application of CNQX (10 μM) and APV (50 μM; $n = 3$) or by TTX (1 μM; $n = 2$). Therefore, repeated applications of kainate to the hippocampus induced persistent changes in the hippocampal network activity.

Discussion

The present study provides several novel observations on the effects of kainate in the developing limbic system *in vitro*. Our results suggest that: (i) hippocampal seizures build up during the first postnatal week in parallel with the development of postsynaptic responses to kainate in CA3 pyramidal neurons; (ii) the hippocampus is the primary site of action of kainate in the limbic system and the intralimbic propagation of seizures is age-dependent; (iii) seizures produce persistent alterations in the GDPs that may be deleterious in spite of the relative resistance of neurons to the epileptogenic actions of kainate (Nitecka *et al*., 1984). These results provide a new and exciting insight into the hitherto unsuspected consequences of epileptogenic activities in the neonatal brain. They also reflect the advantages of the intact *in vitro* preparation and the use of the three-compartment chamber to facilitate the study of the molecular and cellular mechanisms of neonatal epileptogenesis.

Developmental profile of hippocampal seizures

The first postnatal week is characterized in the rat by major changes in neuronal activity and a developmental sequence of alterations in voltage- and transmitter-gated ionic channels that presumably play a crucial role in neuronal growth and formation of the network (reviewed in Ben-Ari *et al*., 1997). During this period, dendrites and axons of CA3 pyramidal cells extensively grow and establish synaptic connections, including presumably recurrent collaterals. The pattern of spontaneous activity during the first week is characterized by the presence of network-driven GDPs that provide most of the activity (Ben-Ari *et al*., 1989; Leinekugel *et al*., 1997, 1998).

Electrographic and metabolic autoradiography studies using the 2-deoxyglucose labelling suggest that in P3 rats, parenteral administrations of kainate generate epileptiform activities that are initially restricted to the CA3 hippocampal region and septum (Tremblay *et al*., 1984). At later developmental stages, other limbic structures are sequentially activated including the entorhinal cortex and at a subsequent stage the amygdaloid complex (this paper). Our data suggest that there is a parallel developmental profile of kainate receptors in CA3 pyramidal neurons and the epileptogenic actions of kainate. At P0, kainate generated either little or no current in CA3 pyramidal neurons and no epileptiform activities. At P4, large currents and ictal discharges were observed. As in adult slices (Castillo *et al*., 1997; Vignes & Collingridge, 1997), these CNQX-sensitive currents are evoked in the presence of a selective AMPA receptor antagonist suggesting that they are mediated by kainate receptors. Therefore, the epileptiform discharges are triggered by the

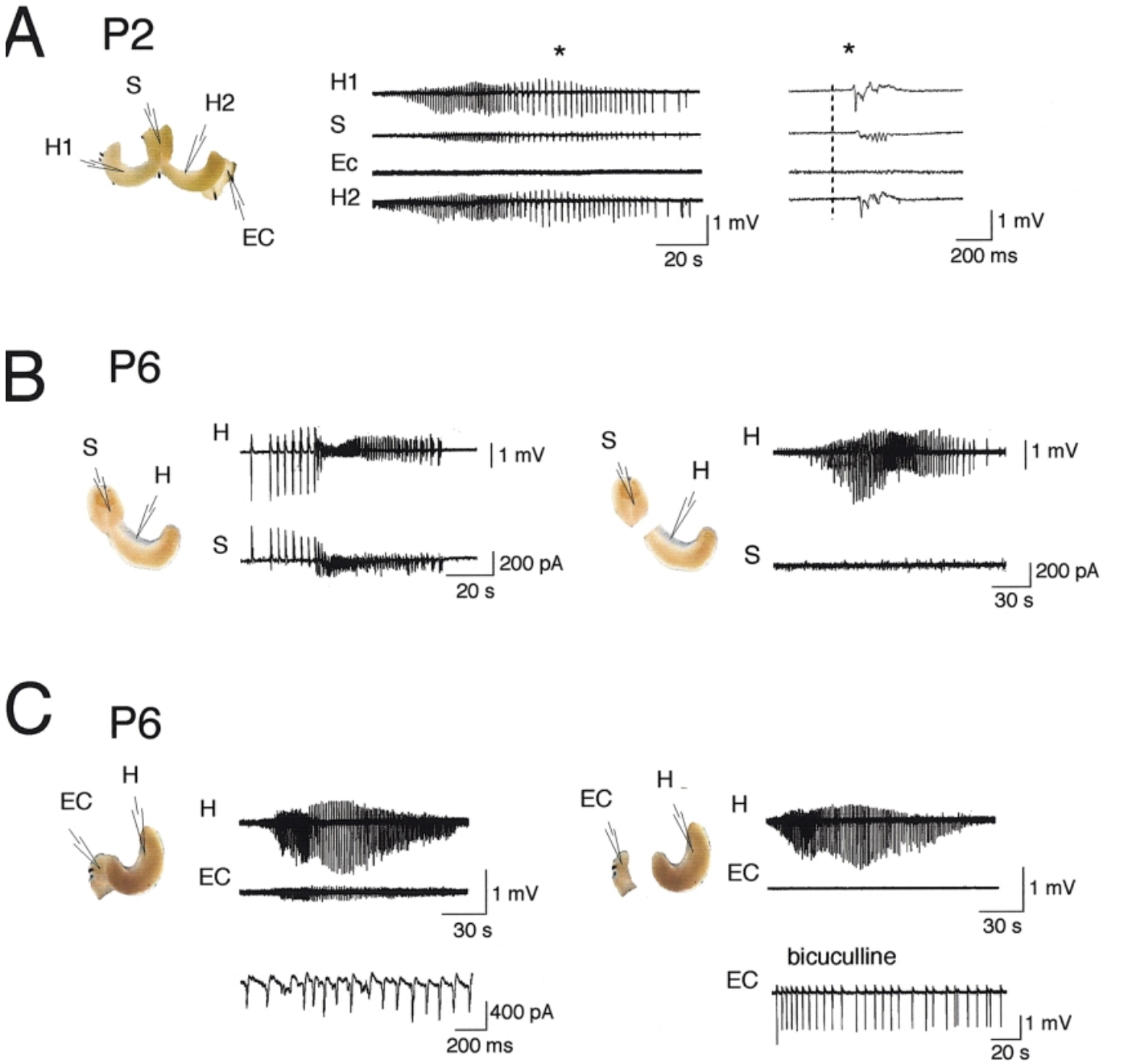

FIG. 8. Propagation of the epileptiform activity from the hippocampus to septum and entorhinal cortex. (A) Photomicrograph of the two hippocampi (H1 and H2), septum (S) and entorhinal cortex (EC) and the positions of the field recording electrodes. At P2, kainate (300 nM) induced synchronized interictal activities in both hippocampi that propagated to the septum but not entorhinal cortex. An interictal event marked by the asterisk is shown on the right on an expanded time scale. Note the delay of propagation from hippocampus to septum. (B) In P6 septo-hippocampal complex, the propagation of epileptic activity from hippocampus to septum (left) was prevented by cutting the connections between the structures (right). Simultaneous field hippocampal recording and whole-cell recording of a septal neuron with a K-gluconate pipette solution at a holding potential of –60 mV. (C) In P6 hippocampo-entorhinal complex, seizures partly propagate from hippocampus to the entorhinal cortex (left). Hippocampal and EC activities are recorded with extracellular electrodes. On the lower trace, whole-cell, voltage-clamp recording from a layer 5–6 neuron in EC during the tonic phase of ictus. On the right, epileptic activity was generated in the hippocampus but not in EC after cutting the connections between the structures (upper traces). However, application of bicuculline (10 μM) to the disconnected EC triggered epileptiform activity (lower trace, extracellular recordings).

activation of postsynaptic high-affinity kainate receptors in CA3 pyramidal cells. Interestingly, in the adult hippocampus, binding (Foster *et al*., 1981; Represa *et al*., 1987; Dessi *et al*., 1991), morphological (Gaiarsa *et al*., 1992, 1994) and electrophysiological studies (Fisher & Alger, 1984; Sawada *et al*., 1988; Castillo *et al*., 1997; Vignes & Collingridge, 1997) suggest that kainate receptors located on mossy fibre synaptic inputs to CA3 pyramidal neurons are the principal target of kainate to generate limbic epileptiform activities (reviewed in Ben-Ari, 1985). Furthermore, kainate generates neither inward currents in CA3 pyramidal neurons nor limbic seizures in GluR6 (glutamate receptor) -deficient mice (Mulle *et al*., 1998), suggesting that kainate receptors containing this subunit are involved in the epileptiform actions of kainate. Although the present results strongly suggest that activation of the postsynaptic

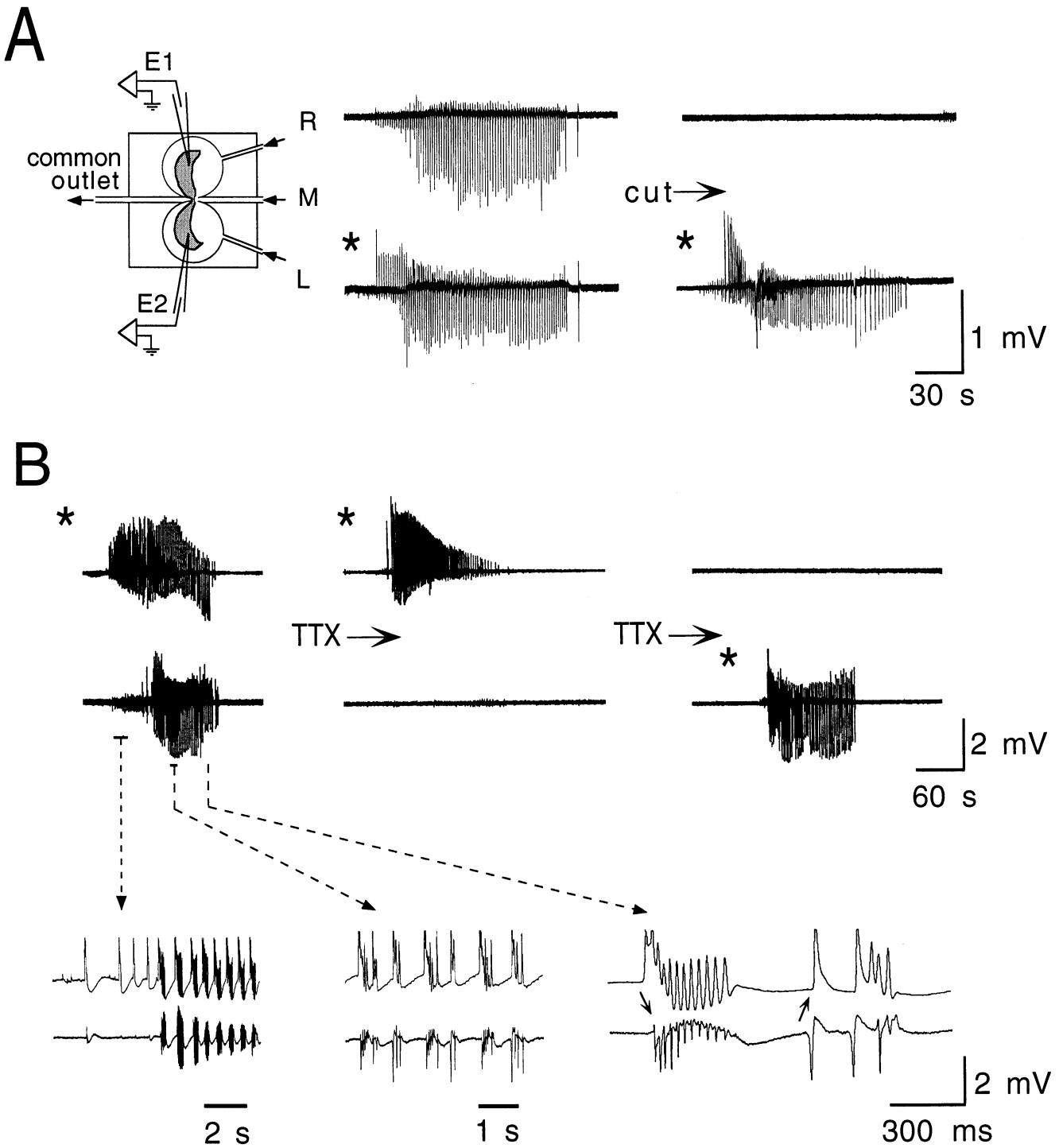

FIG. 9. Propagation of the epileptiform activity from ipsilateral to contralateral hippocampus via commissural connections. (A) Three-compartment chamber: the two hippocampi and the commissural connections have independent inlets and a common outlet allowing independent perfusion of each hippocampus and commissure. At P2, application of kainate (300 nM) to one (ipsilateral) hippocampus (the side of the application is marked by the asterisk) generates an interictal-like seizure activity that propagates to the contralateral side (left traces). Cutting the commissural connections prevents the contralateral propagation of seizure activity. (B) At P6, kainate-induced ictal-like activity in the ipsilateral hippocampus propagates to the contralateral hippocampus. Blockade of the commissural connections by application of TTX to the middle compartment or by local pressure ejection of TTX in the commissure reversibly blocks the contralateral propagation of the epileptic activity (middle and right traces). On the lower traces, the co-ordination of the epileptiform activities in the ipsilateral and contralateral hippocampi are shown on an expanded time scale. Note that seizures are initially restricted to the ipsilateral side, then start to propagate to the contralateral side, and finally the contralateral side becomes an epileptogenic focus.

kainate receptors in CA3 pyramidal cells underlies the epileptogenic action of exogenous bath-applied kainate, it remains to be elucidated whether synaptic activation of kainate receptors by endogenous glutamate released by mossy fibres or via spillover of glutamate from the recurrent collateral synapses contributes to the synchronization of the epileptiform discharges. It will also be important to determine the

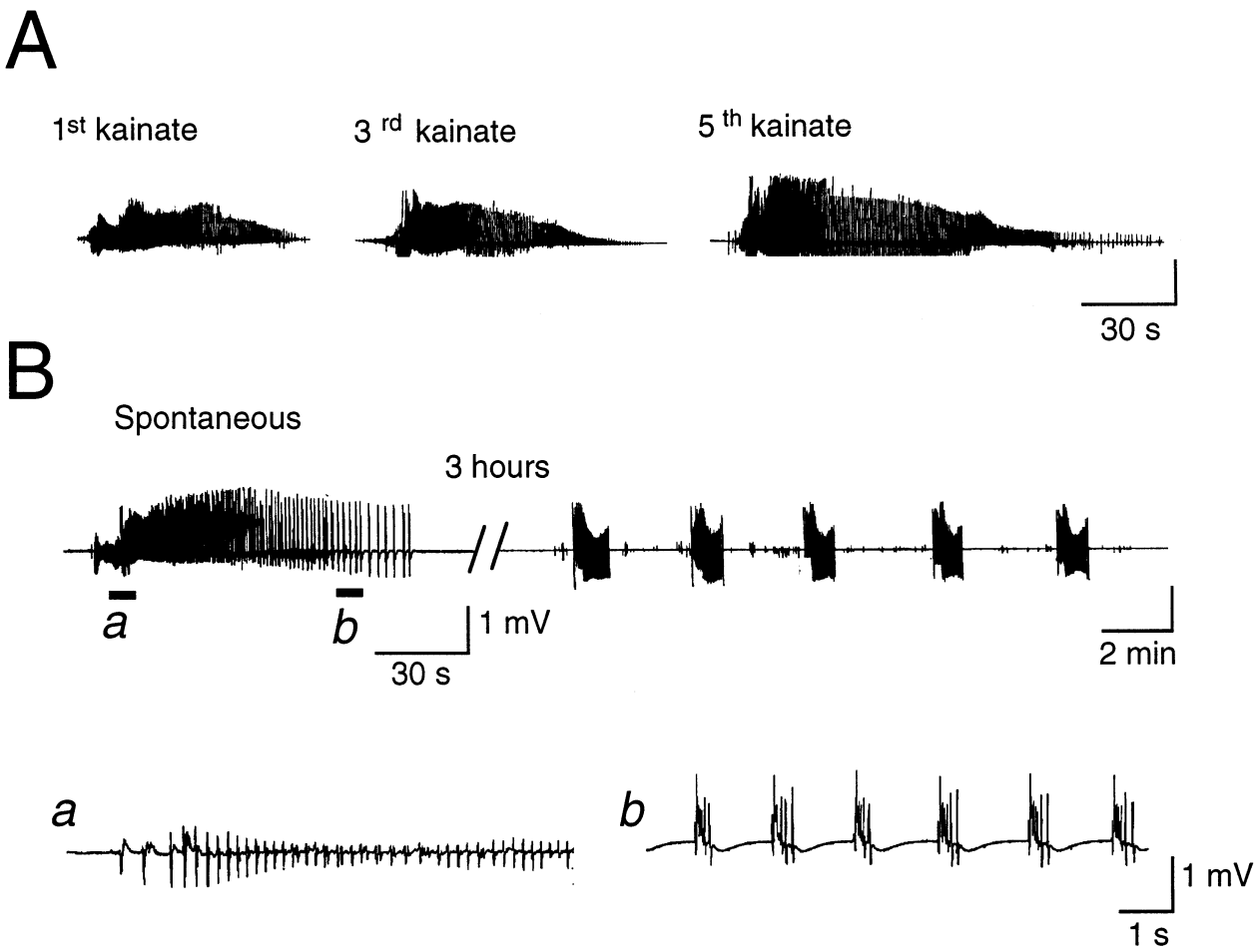

FIG. 10. Long-term effect of kainate (P6 IHF). (A) With repetitive applications of kainate (300 nM), the amplitude and duration of field-recorded paroxysmal discharges progressively increased (10–15 min intervals between applications). (B) After the fifth application of kainate, persistent spontaneous recurrent seizures were generated in the hippocampus. These spontaneous discharges had tonic (*a*) and clonic (*b*) phases.

maturation of the effects of kainate on GABAergic interneurons. Recent studies (see Cossart *et al*., 1998; Frerking *et al*., 1998) indicate that kainate, probably acting on GluR5 subunits (Cossart *et al*., 1998), increases the spontaneous inhibitory tone that impinges on pyramidal neurons, raising the possibility of a dual action of kainate that will generate epileptiform activities via its action on pyramidal neurons and reduce the threshold of seizures via an increase of tonic inhibition (Cossart *et al*., 1998).

Another determining feature is the development of the recurrent collaterals that interconnect CA3 pyramidal neurons and play a pivotal role in the synchronization of the epileptic discharges (Miles & Wong, 1986; Traub *et al*., 1987; Smith *et al*., 1995). Our observation that glutamatergic ictal and interictal activities can be generated a few days after birth suggests that recurrent collaterals are operational at an early developmental stage (see also Gomez-Di Cesare *et al*., 1997). In parallel studies, ictal discharges were generated by other major convulsant drugs including bicuculline, high K^+ and 4-AP (4-amino-pyridine) in the neonatal intact hippocampus preparation (Khalilov *et al*., 1997a,b; Luhman, H., Dzhala, V. & Ben Ari, Y., unpublished results). Interestingly, selective AMPA and NMDA receptor antagonists blocked the epileptiform activities suggesting that, as in the adult hippocampus (Foster *et al*., 1981; Represa *et al*., 1987; Sawada *et al*., 1988; Dessi *et al*., 1991; Gaiarsa *et al*., 1992, 1994; Castillo *et al*., 1997; Vignes & Collingridge, 1997), neonatal recurrent collateral synapses are mediated by AMPA-NMDA receptors, not kainate receptors.

Present data indicate that ictal activities can be generated much earlier than previously suggested on the basis of slice data (Swann & Brady, 1984; Swann, 1995; Swann & Moshe, 1997). Three points are worth stressing: (i) most earlier studies have concentrated on P5 and more mature slices, but the abrupt changes that take place during the first week indicate that this period is by no means a single homogeneous entity; (ii) the developmental profile of the epileptiform activity generated by kainate in the intact *in vitro* preparation is in excellent agreement with *in vivo* data because severe seizures are already observed 3 days postnatally (Cherubini *et al*., 1983; Tremblay *et al*., 1984); (iii) conclusions derived from slice studies in neonates may be particularly handicapped by the loss of intrinsic and external inputs because of the paucity of connections and the need for a minimal mass of neurons and synaptic inputs in order to generate an ictal discharge (Smith *et al*., 1995). Other reasons are better conditions for extrasynaptic and field interactions and the accumulation of extracellular potassium (Jefferys, 1995). The intact preparation may be more relevant than slices for studying network-driven activities (Khalilov *et al*., 1997b).

Propagation of seizures in the developing limbic system

Previous studies using 2-deoxyglucose autoradiography have suggested that, in the neonatal rat, the hyperactivity generated

by parenteral injections of kainate is restricted to the hippocampi and septum at P3 and subsequently propagates to other limbic structures (Nitecka *et al*., 1984; Tremblay *et al*., 1984). Present results show indeed that *in vitro* seizures are initially restricted to hippocampi and septum suggesting that commissural and hippocampo-septal connections develop first and become functional a few days after birth, in keeping with morphological data using tracing techniques *in vivo* (Super & Soriano, 1994; Linke *et al*., 1995). Propagation of the hippocampal seizures to the entorhinal cortex is observed later, at the end of the first postnatal week. Altogether these data suggest that indeed the hippocampus is a primary site of the action of kainate and that the intralimbic propagation of the hippocampal seizure is age-dependent. Using the same *in vitro* preparation of limbic structures, we have found in a parallel study that already at P2, GDPs propagate from the hippocampus to the septum (Leinekugel *et al*., 1998). These observations suggest that the hippocampus operates as a pacemaker region during the maturation of the limbic system and is in a position to modulate its development by activity-dependent mechanisms. A long-lasting perturbation of hippocampal activity may therefore have deleterious consequences on the formation of the limbic circuitry.

Long-lasting consequences of kainate-induced seizures

In keeping with this conclusion, repeated applications of kainate to neonatal limbic structures blocked the GDPs and generated recurrent spontaneous epileptiform activities. These effects persisted for several hours and were limited by the survival period of the preparation, suggesting that they may persist for even longer duration *in vivo*. In the adult, synchronized activities occur in different behavioural conditions and participate in neural plasticity (Buzsaki & Chrobak, 1995; McCormick & Bal, 1997; Steriade, 1998). Evidence is also accumulating that synchronized patterns of activity such as the GDPs that predominate during development (see Yuste *et al*., 1995; Feller *et al*., 1996; Leinekugel *et al*., 1997) are instrumental in plasticity and the formation of functional neuronal networks during development (Goodman & Shatz, 1993). Alterations in these patterns may be detrimental and provide the basis for the well-known clinical and experimental paradox that neonatal seizures have long-term sequelae yet immature neurons are far more resistant to the pathological effects of repeated seizures (Ben-Ari, 1985; Holmes, 1997; Holmes & Ben-Ari, 1998). They may lead to persistent alterations in the developmentally-regulated activity-dependent expression of receptors as shown in a variety of systems (Xie & Ziskind-Conhaim, 1995). The alteration of the GDPs, which are a major feature of developing networks and which have been observed in most immature structures studied (see Ben-Ari *et al*., 1997), will have profound consequences on the development of the network. It may alter the formation of appropriate connections according to the concept that 'cells that fire together wire together' (Goodman & Shatz, 1993). A recent study suggests that repeated seizures in the neonatal rat lead in adulthood to a sprouting of mossy fibres, and that the establishment of novel aberrant mossy fibre synapses is associated with a reduction in the threshold for seizures and severe deficits in learning tests (Holmes *et al*., 1998; reviewed in Holmes, 1997 and Holmes & Ben-Ari, 1998).

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Abbreviations

2-DG, 2-deoxyglucose; ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APV, 2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; EPSC, excitatory postsynaptic current; GABA, γ-aminobutyric acid; GDP, giant depolarising potential; GYKI 56355, AMPA receptor antagonist; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; IHF, intact hippocampal formation; IPSC, inhibitory post-synaptic current; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; P, postnatal day; TLE, temporal lobe epilepsy; TTX, tetrodotoxin.

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