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Persistent epileptiform activity induced by low Mg^{2+} in intact immature brain structures

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Abstract

We have determined the properties of seizures induced *in vitro* during the first postnatal days using intact rat cortico-hippocampal formations (CHFs) and extracellular recordings. Two main patterns of activity were generated by nominally Mg^{2+}-free ACSF in hippocampal and cortical regions: ictal-like events (ILEs) and late recurrent interictal discharges (LRDs). They were elicited at distinct developmental periods and displayed different pharmacological properties. ILEs were first observed in P1 CHFs 52 ± 7 min after application of low-Mg^{2+} ACSF (frequency 1.5 ± 0.3 h^{-1}, duration 86 ± 3 s). There is a progressive age-dependent maturation of ILEs characterized by a decrease in their onset and an increase in their frequency and duration. ILEs were abolished by D-APV and Mg^{2+} ions. From P7, ILEs were followed by LRDs that appeared 89 ± 8 min after application of low-Mg^{2+} ACSF (frequency ≈ 1 Hz, duration 0.66 s, amplitude 0.31 ± 0.03 mV). LRDs were no longer sensitive to D-APV or Mg^{2+} ions and persisted for at least 24 h in low-Mg^{2+} or in normal ACSF. ILEs and LRDs were synchronized in limbic and cortical regions with 10–40 ms latency between the onsets of seizures. Using a double chamber that enables independent superfusion of two interconnected CHFs, we report that ILEs and LRDs generated in one CHF propagated readily to the other one that was being kept in ACSF. Therefore, at a critical period of brain development, recurrent seizures induce a permanent form of hyperactivity in intact brain structures and this preparation provides a unique opportunity to study the consequences of seizures at early developmental stages.

Introduction

Animal models of epilepsy developed in immature brain slices have provided valuable information about the characteristics of seizures generated by convulsive agents in these tissues but suffer from severe limitations which restrict their extension to human childhood epilepsies. Thus, at a time when neurons are poorly developed, the slicing procedure removes many inputs and seriously modifies the basic activity. Furthermore, within the thickness of the slice, fewer synapses are available for the expression of the epileptiform activity (Jefferys, 1998) than in the intact tissue *in vivo*. Accordingly, ictal-like activity has seldom been reported during the first week of life. However, this period is characterized by an intense maturation of neurons that have established their initial contacts (Tyzio *et al*., 1999) and by early functional networks in which GABA is excitatory (Cherubini *et al*., 1991). Alteration of these activity-dependent processes during this critical period of brain development may have adverse consequences which may also be the support of infantile epilepsies (Roger *et al*., 1992). In addition, seizures usually induced by exogenous convulsive agents (bicuculline, kainic acid, 4-aminopyridine etc.) correspond most frequently to acute seizures that disappear when the convulsive agent is removed. This stands in contrast to the long-term alterations that only occur after recurrent seizures and that condition the study of the syndromic evolution of the pathology.

In the present study, we have taken these factors into consideration in order to develop an *in vitro* animal model of infantile epilepsy. Accordingly, we have used the recently developed preparation of intact hippocampus (Khalilov *et al*., 1997a) that offers an excellent compromise between *in vivo* and *in vitro* preparations and allows the study of epileptic processes during the first week of life (Khalilov *et al*., 1997b; Khalilov *et al*., 1999; Luhmann *et al*., 2000). We extended this preparation to nonlimbic areas by including a large part of the posterior cortex, giving an intact cortico-hippocampal formation (CHF) in which the integrity of the network is preserved within and between brain regions. Preliminary studies with several convulsive agents revealed an age-dependent modification of seizures induced by low-Mg^{2+} artificial cerebrospinal fluid (ACSF), largely investigated in adult cortico-hippocampal slices (reviewed in Heinemann *et al*., 1993).

We report that superfusion of intact CHFs with low-Mg^{2+} ACSF generated spontaneous ictal-like events (ILEs) that were synchronized in the limbic and cortical areas. ILEs modify themselves by an age-dependent process leading to interictal late recurrent discharges (LRDs) with completely different pharmacological characteristics. This pattern of activity persisted for long periods even when the physiological concentration of Mg^{2+} ions was restored, suggesting that this preparation represents a new model of epilepsy.

Materials and methods

All protocols were designed according to INSERM guidelines for the care and use of animals. Experiments were performed on the intact CHF taken from Wistar rats between embryonic day (E)19 and postnatal day (P)10. E0 was determined by the presence of spermatozoids in the vaginal plug and P0 was the day of birth. In

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few experiments, P7–8 mice were also used. Briefly, animals were killed and brains were extracted from the skull at 4 °C. The two hemispheres were separated and dissected in order to obtain CHFs. The CHF contains the hippocampus and the septum and approximately the posterior half of the cortex, including the entorhinal cortex and a large part of the neocortical areas. Each CHF was placed in oxygenated (95% O2 and 5% CO2) ACSF with the following composition (in mM): NaCl, 126; KCl, 3.5; $CaCl_2$, 2; $MgCl_2$, 1.3; $NaHCO_3$, 25; $NaHPO_4$, 1.2; glucose 10 (pH 7.3). After at least 1 h rest at room temperature, the CHF was transferred to the recording chamber where it was fully submerged and superfused with oxygenated ACSF at 31 ± 1 °C at a flow rate of 5.0 ± 0.2 mL/min. After 15–30 min, CHFs were then superfused with an ACSF without magnesium ions. In these conditions, the extracellular concentration of Mg^{2+} ions does not drop to zero because the contamination by Mg^{2+} of the other constituents of the ACSF could reach 0.08 mM (Mody *et al*., 1987). Therefore, according to these authors, we use the term low-Mg^{2+} ACSF rather than zero-Mg^{2+} ACSF.

Extracellular recordings

Bipolar twisted nichrome electrodes were used to stimulate Schaffer collaterals and the perforant path (15–30 V, 30 μs duration at 0.033 Hz). Recordings were performed with extracellular electrodes filled with ACSF, usually placed in the CA1 area of the hippocampus at a location of the highest field potential response (Diabira *et al*., 1999). Multiple recordings were also performed in different hippocampal areas (CA1, CA3, dentate gyrus) and in the entorhinal cortex. Field potentials were acquired using a Digidata 1200B (Axon Instruments, CA, USA) card. Data were analysed using Clampfit (Axon laboratories, CA, USA) software.

Drugs

Bicuculline methochloride, 6-cyano-7-nitro quinoxaline-2,3-dione (CNQX), D-2-amino-5-phosphono-pentanoic acid (D-APV), 1-[4-aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7-sulphonamide (NBQX) and isoguvacine were purchased from Tocris Cookson Ltd (UK); other chemical were purchased from Sigma-Aldrich (France). Drugs were first dissolved in water or in DMSO and then diluted in ACSF. The final concentration of 0.1% DMSO has no effect on seizures. All drugs were applied by superfusion (5 mL/min; 31 ± 1 °C).

Pharmacological studies

The effects of drugs were evaluated in the CA1 area of the hippocampus at several concentrations according to the following procedures. (i) Effects on ILEs: following the expression of the third ILE, drugs were superfused over 30 min and then washed out. When a drug prevented the emergence of ILEs, we tested at the end of its application period whether electrical stimulation (60–80 V, 30 μs or tetanus 20 Hz, 5 s, 60 μs) could evoke ILEs. (ii) Effects on LRDs: after recording an initial period of stable LRDs (30 min), drugs were applied over 30 min and their effects on amplitude and frequency were compared to the control situation.

Anoxic–aglycemic experiments

Episodes of 5 min anoxia were induced by superfusion with low-Mg^{2+} ACSF saturated with 95% nitrogen and 5% oxygen. An equimolar concentration of sucrose was substituted for glucose in the low-Mg^{2+} ACSF to aggravate the metabolic stress (anoxic ACSF).

Statistical analysis

Results are expressed as mean values ± SEM of n independent experiments. One-way ANOVA was used for significance determination: $P < 0.05$ was considered a significant difference between mean values.

Results

Age-dependent seizures induced by low-Mg^{2+} ACSF

Intact CHFs taken from E19–P10 rats (Fig. 1A) were continuously superfused for several hours with an ACSF from which Mg^{2+} ions had been omitted (low-Mg^{2+} ACSF) and recorded with extracellular electrodes placed in the CA1 area. This procedure generated different patterns of hyperactivity according to the age of the rats (Fig. 1B, Table 1). (i) Only irregular interictal events of small amplitude were observed in E19–P0 rats (n = 8, Fig. 1B). (ii) Starting from P1 (n = 6), low-Mg^{2+} ACSF consistently generated ILEs that were present as long as the low-Mg^{2+} ACSF was maintained (Fig. 1B, Table 1). The onset of the first ILEs progressively reduced with age whereas amplitude, duration and frequency increased (Fig. 1B, Table 1). (iii) After P6, in addition to ILEs, low-Mg^{2+} ACSF generated a novel pattern of activity characterized by regular late recurrent interictal discharges generated at a frequency around 1 Hz (LRDs) (Fig. 1C, Table 1).

Thus, low-Mg^{2+} ACSF generated recurrent seizures with a time-dependent pattern associated with two dominating activities, the ILEs and the LRDs. The detailed characteristics of ILEs and LRDs were then more thoroughly studied in P7–8 CHFs (Table 2). Continuous superfusion of CHFs with low-Mg^{2+} ACSF usually generated 5–8 ILEs. Each event consisted of an initial interictal-like burst, a tonic phase (7.9 ± 0.6 s) followed by a long-lasting tonic–clonic period (47 ± 2 s) and finally by the gradual development of recurring clustered bursts separated by short silent intervals of increasing duration (clonic phase: 48 ± 4 s) (Fig. 1C). After 1 h, ILEs were followed by one and occasionally two long-lasting ILEs (480–800 s). The pattern shifted then to LRDs, which persisted subsequently (Table 2). During the period of expression of ILEs, a single electrical stimulus or low-frequency stimulation evoked an ictal-like response similar to spontaneous ILE but during the expression of LRDs all types of electrical stimulation failed to induce an evoked response (not shown). Thus, there was at the end of the first week a shift from one type of interictal pattern to another of more robust recurrent discharges. We then tested the pharmacological differences between the two patterns in P7–8 CHFs.

Pharmacology of low-Mg^{2+}-induced seizures

Both ILEs and LRDs generated in the CA1 area of intact CHFs were network-driven as they were blocked by tetrodotoxin (1 μM, 3 min, n = 6) (Figs 2A and 3A). Testing the effects of various drugs, relying on the procedure described in Materials and Methods, revealed major differences between the two patterns of activity.

Glutamate receptors

As expected, NMDA receptors were involved in the generation of ILEs. Thus, ILEs were completely and reversibly blocked by D-APV (30 μM; n = 8) and no further ILE could be evoked by electrical stimulation or by a low-frequency train of stimulation (Fig. 2B). Likewise, superfusion with a physiological concentration (1.3 mM) of Mg^{2+} ions (n = 10) completely and reversibly blocked the recurrence of ILEs (Fig. 2C). In contrast, CNQX (60 μM; n = 8) reduced the amplitude (–16 ± 3%) and duration (–31 ± 6%) of ILEs but did not

block them (Fig. 2D). Similar results were observed with NBQX (10 μM; $n = 3$) or GYKI 52466 (30 μM; $n = 3$) (not shown).

A different pharmacological profile was observed for LRDs. D-APV (60 μM; $n = 12$; Fig. 3B) did not reduce LRDs; in fact the amplitude was increased (by up to 40%) in 12 out of 16 experiments with D-APV (20–60 μM). Likewise, LRDs were not reduced by physiological concentrations of Mg^{2+} ions ($n = 16$). There was occasionally an increase in the amplitude of LRDs and a reduction in duration of (–23 ± 5%; Fig. 3C). AMPA/KA receptors played a more important role in the expression of LRDs than did NMDA receptors as CNQX at a relatively low concentration (10 μM; $n = 6$) reduced by 69 ± 4% the amplitude of LRDs. A higher concentration of CNQX (60 μM; $n = 3$) did not consistently improve this blockade (Fig. 3D) but superfusion with CNQX (10 μM) and D-APV (30 μM; $n = 4$) completely suppressed LRDs (Fig. 3D). The characteristics of this partial blockade have not been further investigated. However, it may be related to paradoxical effects of CNQX (Geoffroy *et al.*, 1991; McBain *et al.*, 1992) rather than to its AMPA receptor antagonist properties.

Therefore, glutamate antagonists differentially affect the early and the late phases of hyperactivity. NMDA receptors were required for the induction of ILEs whereas AMPA receptors appeared to be involved in the maintenance of the late epileptiform activity.

$GABA_A$ receptors

Spontaneous and evoked ILEs were blocked by relatively high concentrations (100 μM; $n = 8$) of the $GABA_A$ agonist isoguvacine (Fig. 2E). Lower concentrations either had no significant effect (10 μM; $n = 4$) or did not prevent the induction of evoked ILEs (60 μM; $n = 8$). In contrast, LRDs were completely blocked, in a reversible manner, by a low concentration of isoguvacine (10 μM; $n = 6$) (Fig. 3E). Therefore, LRDs were more efficiently prevented by activation of $GABA_A$ receptors than were ILEs. Application of bicuculline (10 μM; $n = 12$) during ILEs generated bursts of interictal discharges (Fig. 2F) that persisted after washout of the $GABA_A$ antagonist. Application of bicuculline during the expression of LRDs (10 μM; $n = 6$) generated ictal-like events (Fig. 3F), suggesting that inhibition was still partly operative during ILEs and LRDs.

Induction and propagation of low-Mg^{2+}-induced seizures

Induction

Electrodes were placed in the stratum radiatum of CA1 and in the entorhinal cortex of P7–8 rats ($n = 12$). Low-Mg^{2+} ACSF induced a synchronized pattern of activity in the two brain regions but the amplitudes of ILEs and LRDs were less marked in the entorhinal cortex than in the hippocampus (Fig. 4A). The initial ILE occurred either in the hippocampus ($n = 7$) or in the entorhinal cortex ($n = 5$) but the delays between the two onsets were in the range 10–40 ms. In contrast, the late clonic phases of ILEs had an almost simultaneous generation in both regions. To confirm that seizures could be generated in the hippocampus proper, cortical areas were separated from the hippocampus by surgical cuts that disrupted reciprocal afferent fibres and the isolated intact hippocampus was superfused with low-Mg^{2+} ACSF. A full pattern of epileptic activity ($n = 8$) with ILEs followed by LRDs was generated in the isolated hippocampus (not shown). The onset of the first ILE (19.6 ± 1.1 min) and of LRDs (104 ± 7 min) were not significantly different from those reported in intact CHFs (Table 2). Furthermore, to examine whether a particular region of the hippocampus could be involved in the generation of seizures, electrodes were placed in stratum radiatum of CA1 and CA3 and in the granular cells of the dentate gyrus ($n = 8$). ILEs and LRDs

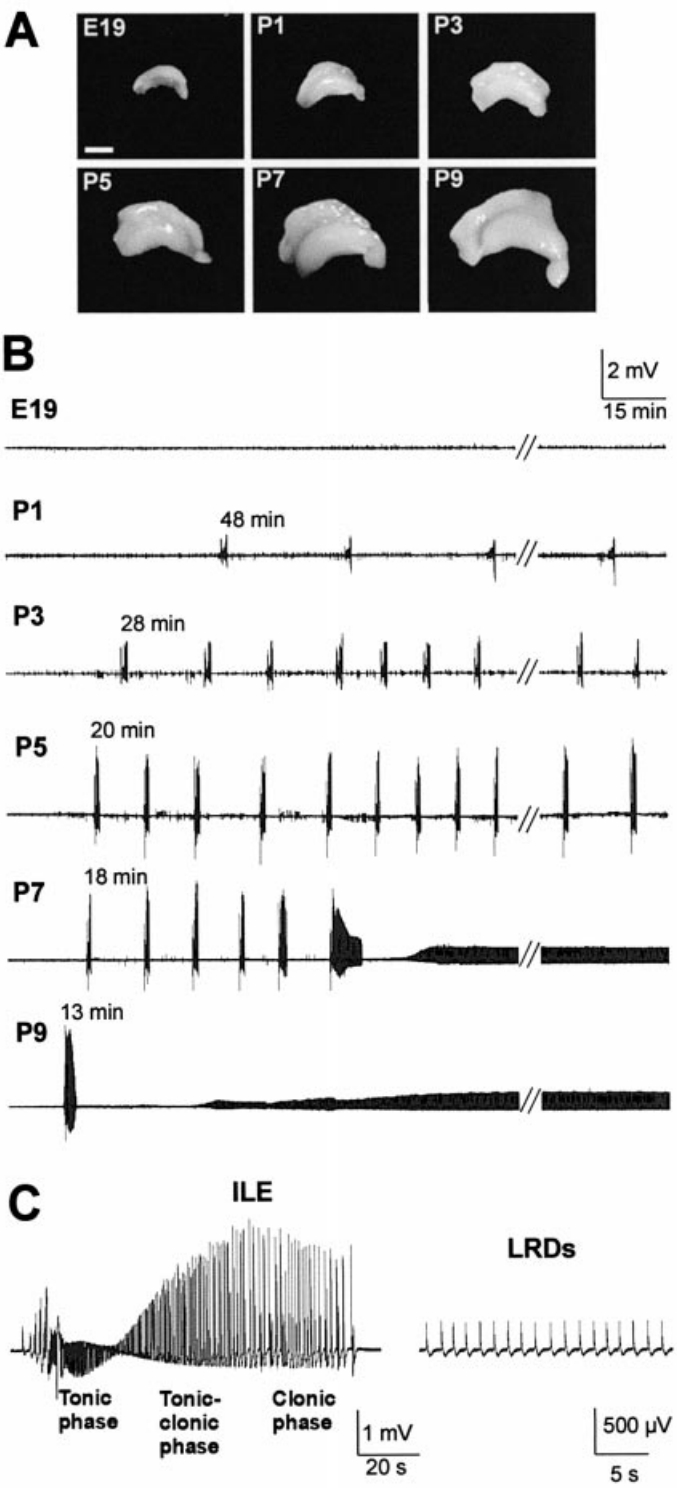

FIG. 1. Age-dependent effect of low-Mg^{2+} ACSF. (A) Photos of intact CHFs taken from E19–P9 rats. (B) Three types of hyperactivity were induced in CHFs continuously superfused with nominally Mg^{2+}-free ACSF and recorded in CA1 with extracellular electrodes. Only an irregular interictal-like activity was recorded at E19. Repetitive ILEs were generated in P1, P3 and P5 CHFs. The value above the first ILE indicated the time elapsed from the beginning of the superfusion with low-Mg^{2+} ACSF. Their frequency, amplitude and duration increased with age. At P7 and P9, ILEs were generated first and, after 60–90 min, another pattern of discharges (LRDs) appeared. (C) Enlargements of the first ILE generated and of LRDs occurring in P7 CHFs. Note that traces are interrupted (//) for 1 h. Scale bar in (A), 2.5 mm.

TABLE 1. Age-dependence of low-Mg^{2+}-ACSF-induced seizures in intact CHFs

		First ILE			ILE frequency	LRDs	
Age range	*n*	Onset (min)	Amplitude (mV)	Duration (s)	(per hour)	Onset (min)	Frequency (per second)
E19–PO	8	no ILE	–	–	–	no LRDs	–
P1–2	10	52 ± 7	0.55 ± 0.06	86 ± 3	1.5 ± 0.3	no LRDs	–
P3–4	8	27 ± 3	0.94 ± 0.23	92 ± 8	3.5 ± 0.5	no LRDs	–
P5–6	21	23 ± 2	1.12 ± 0.10	98 ± 4	4.1 ± 0.4	no LRDs	–
P7–8	94	19.7 ± 0.8	1.30 ± 0.05	112 ± 6	4.2 ± 0.3	89 ± 8	0.72 ± 0.07
P9–10	7	13.9 ± 2.7	1.22 ± 0.11	179 ± 2	–	55 ± 5	1.15 ± 0.15

E19–P10 CHFs were continuously superfused with nominally Mg^{2+}-free ACSF (see Materials and methods). Ictal-like events (ILEs) and late recurrent discharges (LRDs) were recorded in the CA1 area of intact CHFs. Note that no ILE was recorded before P1 and that LRDs started at P7. At P9–10, only one or two ILEs were expressed before LRDs.

appeared to be synchronized within these regions and again no leading region appeared and the delays for the generation of ILEs between different hippocampal regions never exceeded 40 ms (Fig. 4B). Thus, there was no preferential site of generation of the ILEs and LRDs in intact CHFs.

Propagation

In order to dissociate generation of seizures from their propagation, we used two intact CHFs that were kept interconnected through the commissure (Fig. 5A) (Khazipov *et al*., 1999). The two CHFs were placed in two chambers in which the flow rate and the position of outlets were adjusted to prevent any exchange of buffer between the two compartments (Fig. 5B). As reported previously (Khazipov *et al*., 1999), we checked by spectrometric detection of a dye (methylene blue) that there was no significant communication between the two compartments (Fig. 5B). Therefore, this preparation was used to perfuse one CHF (ipsilateral side) with low-Mg^{2+} ACSF whereas the second one (contralateral side) was superfused with physiological ACSF. Both ILEs and LRDs propagated readily from one side to the other (Fig. 5C, $n = 9$). An inter-CHF delay of 1.2–1.5 s (1.39 ± 0.07 s) between the two temporal poles of the hippocampus allowed an estimation of the velocity of propagation as 10–15 mm/s, assuming that the length of one hippocampus was 11 mm and the distance between the two recording electrodes was 16–18 mm. Because ILEs were usually not expressed in physiological ACSF, these results suggest that induction and propagation of seizures are sustained by different mechanisms.

Characteristics of LRDs in intact CHFs

Long-lasting duration of LRDs

In P7–8 CHFs ($n = 14$), LRDs were largely unaffected after 6–8 h (Fig. 6A, a) and were not reversed by 1-h application of physiological concentrations of magnesium ions (Fig. 6A, b). Tissues were then transferred from the perfusion chamber to a well oxygenated solution of 250 mL low-Mg^{2+} ACSF maintained at 30 °C for 16–18 h. Recording again in the CA1 area of the hippocampus revealed an epileptiform activity in most CHFs (12 out 14). The continuous pattern of interictal discharges was slightly modified after 24 h with a decrease in amplitude (–18 ± 3%) and frequency (–29 ± 6%) (Fig. 6A, c), and these discharges were largely unaffected when the physiological concentration of Mg^{2+} was restored for 1 h (Fig. 6A, d).

In order to evaluate whether LRDs also persisted for long periods in normal ACSF, intact P7–8 CHFs were first incubated with low-Mg^{2+} ACSF until stable LRDs were expressed in the CA1 area of the hippocampus. The low-Mg^{2+} buffer was then changed for ACSF and LRDs were recorded 1 h (Fig. 6B, a) and 6–8 h later (Fig. 6B, b) in the superfusing chamber ($n = 5$). The amplitude of LRDs transiently increased (see above) but slightly decreased after 6–8 h superfusion in ACSF (14 ± 4%, $n = 8$, Fig. 6B, b). Tissues were then transferred from the perfusion chamber to a well oxygenated solution of 250 mL ACSF maintained at 30 °C for 16–18 h. Recording again in the CA1 area of the hippocampus revealed a persistent epileptiform activity in all CHFs (Fig. 6B, c). Compared to LRDs initially expressed in ACSF, amplitude and duration of LRDs were clearly attenuated, by –44 ± 7% and –38 ± 4%, $n = 8$, respectively, whereas the frequency was increased by 19 ± 6%, $n = 8$, after 24 h.

TABLE 2. Characteristics of low-Mg^{2+}-ACSF-induced seizures in P7–8 intact CHFs

Event	*n*	Onset (min)	Amplitude (mV)	Duration (s)
ILE 1	94	19.7 ± 0.8	1.30 ± 0.5	112 ± 6
ILE 2	94	34.6 ± 1.1	1.20 ± 0.08	133 ± 8
ILE 3	94	47.4 ± 0.4	1.17 ± 0.08	160 ± 13
ILE 4	72	57.7 ± 3.4	1.10 ± 0.10	206 ± 32
ILE 5	61	68.9 ± 3.8	0.98 ± 0.16	293 ± 73
ILE 5	48	71.7 ± 7.4	0.90 ± 0.17	508 ± 52
LRDs	48	89 ± 8	0.31 ± 0.03	0.66 ± 0.02

Successive ILEs were induced in low-Mg^{2+} ACSF. The onset, duration and amplitude of each ILE are reported. ILE, ictal-like event; LRDs, late recurrent discharges.

LRDs and anoxia in intact CHFs

Because LRDs have not been reported in slices, we wanted to examine whether LRDs could result from accidental experimental anoxia, which is more easily induced in CHFs than in slices. Given the importance of such a phenomenon we performed a series of experiments that completely excluded this possibility.

We first examined the effect of low-Mg^{2+} ACSF on P7–8 CHFs from mice, which are much smaller than those of age-matched rats and therefore less susceptible to anoxia. A pattern of discharge with a sequential formation of ILEs and LRDs was consistently recorded in the CA1 region of mouse CHFs (Fig. 7A) and the onset of LRDs remained in the same time range (72 ± 5 min, $n = 6$) as reported above for P7–8 rats (89 ± 8 min) (Table 2).

We then examined the direct effects of anoxic–aglycemic episodes on the two patterns of discharges induced in P7–8 rat CHFs by low-Mg^{2+} ACSF. CHFs ($n = 18$) were first allowed to equilibrate in low-

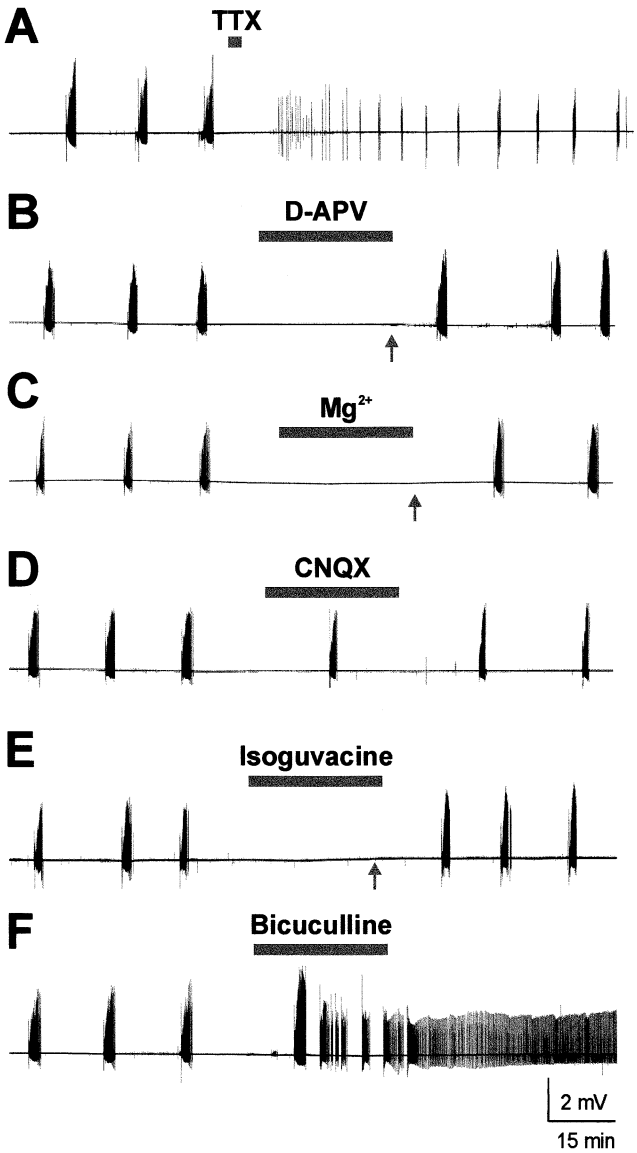

FIG. 2. Pharmacology of low-Mg^{2+} ACSF-induced ILEs at P7–8. ILEs were induced in CHFs continuously superfused with nominally Mg^{2+}-free ACSF and recorded in CA1 with extracellular electrodes. Drugs were superfused after the third ILE. (A) TTX, 1 μM, was applied for 3 min (bar) and washed with low-Mg^{2+} ACSF. (B–F) D-APV (30 μM), Mg^{2+} (1.3 mM), CNQX (60 μM), isoguvacine (100 μM) or bicuculline (10 μM) were superfused over 30 min (bars) and the preparation was washed with low-Mg^{2+} ACSF. Note that an ILE was expressed during CNQX application and that a high concentration of isoguvacine (100 μM) was required to completely block ILEs. The arrow indicates electrical stimulation (80 V, 30 μs).

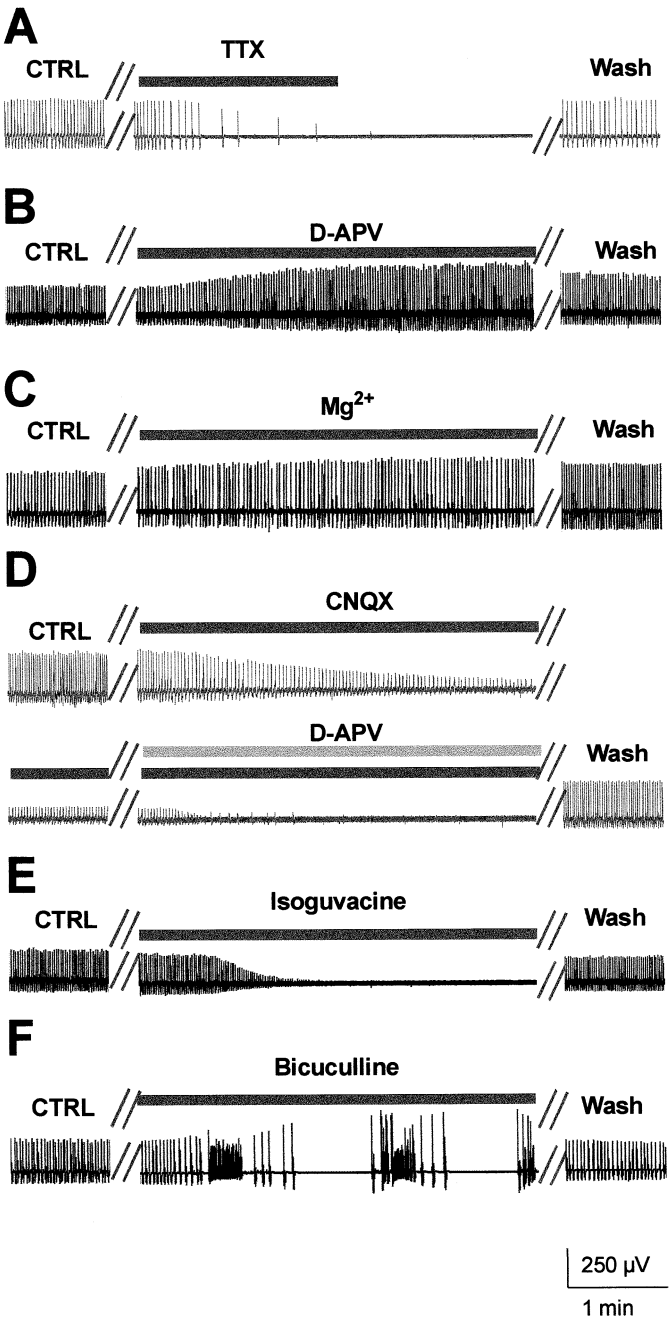

FIG. 3. Pharmacology of low-Mg^{2+} ACSF-induced LRDs at P7–8. Extracellular recordings were performed in the CA1 area of intact CHFs. Stable and regular LRDs were observed over 30 min and drugs were then applied for 30 min except for TTX (3 min). Only 4 min of the application is shown. (A–F) LRDs recorded before (CTRL), during application of TTX (1 μM), D-APV (60 μM), Mg^{2+} (1.3 mM), CNQX (10 μM), CNQX (10 μM) + D-APV (30 μM), isoguvacine (10 μM) or bicuculline (10 μM) (bars) and after washing (wash) with low-Mg^{2+} ACSF. Note that D-APV induced an increase in the amplitude of LRDs. CNQX reduced LRDs that were completely blocked by further addition of D-APV. A lower concentration of isoguvacine (10 μM) was then required to completely block the expression of LRDs.

Mg^{2+} ACSF for 15 min and the anoxic–aglycemic ACSF gassed with nitrogen instead of oxygen was applied for 5 min. Upon re-oxygenation in the low-Mg^{2+} ACSF, several ILEs were elicited (5.5 ± 1.2), followed by interictal discharges which appeared significantly later (155 ± 11 min, $n = 12$, $P = 0.0027$; Fig. 7B, a) than in control CHFs (see Table 2). In 33% of the experiments up to 19 ILEs were recorded over 4 h but LRDs were not observed (not shown). Furthermore, anoxic–aglycemic episodes (5 min) performed during stable expression of LRDs in P7–8 CHFs completely and reversibly suppressed all epileptiform activity (Fig. 7B, b; $n = 6$). Therefore, the expression of LRDs was either delayed or suppressed by anoxic episodes.

In a second set of experiments we examined whether LRDs could be induced in CHFs before P7, either during long-duration superfusion or when CHFs were exposed to anoxia. CHFs from P5 rats were superfused for 24 h with low-Mg^{2+} ACSF. Only ILEs were

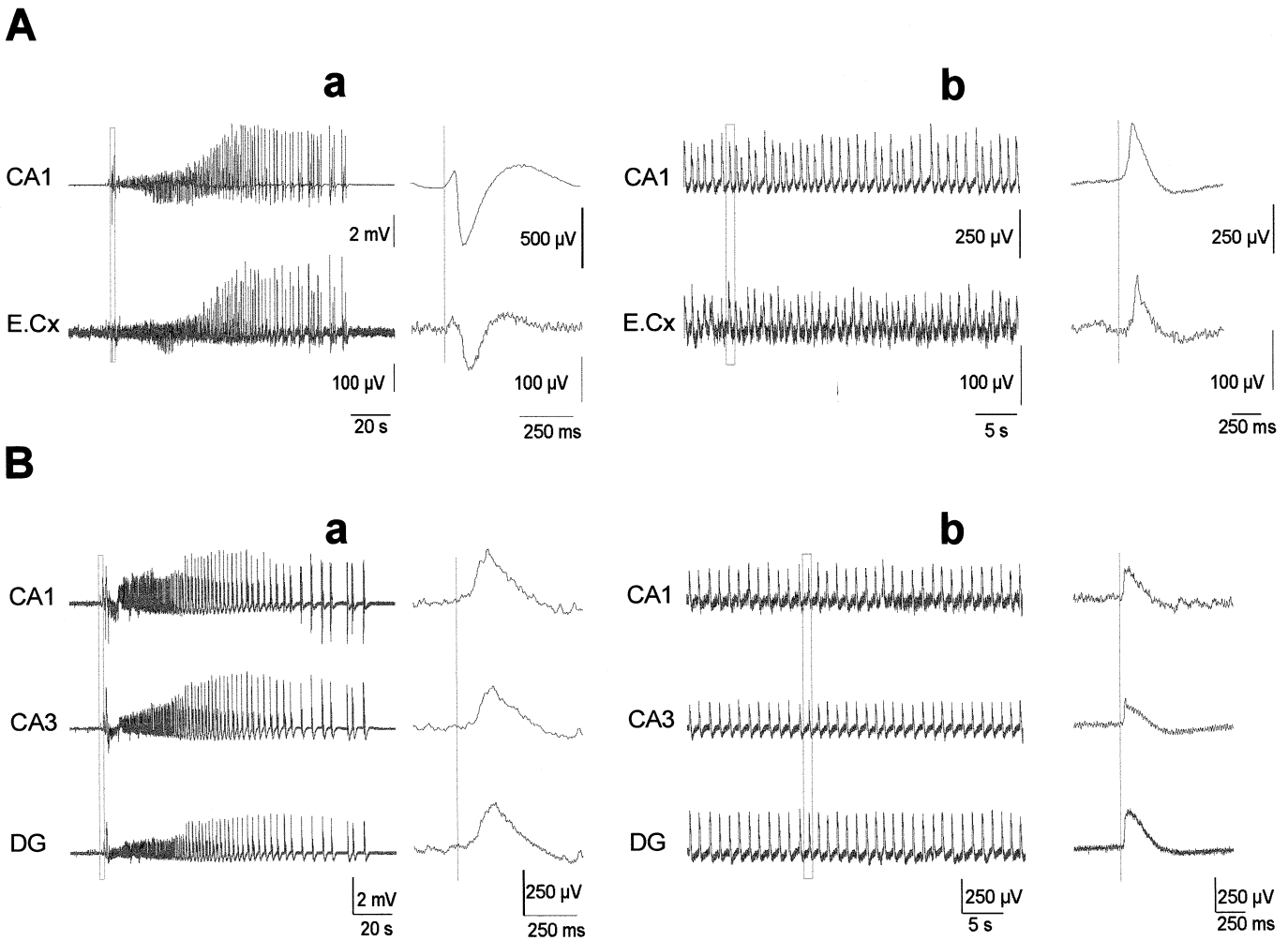

FIG. 4. Synchronization of seizures induced by low-Mg^{2+} ACSF in the hippocampus and entorhinal cortex of P7–8 CHFs. (A) ILEs and LRDs were generated in the CA1 area of the hippocampus (CA1) and entorhinal cortex (E.Cx). The first ILE (a) and LRDs (b) recorded in the two brain areas were synchronized. Note the lower amplitude of cortical records. (B) Extracellular electrodes were placed in CA1, CA3 and dentate gyrus (DG) regions of intact CHFs. Note the small delay between the onset of the first ILE (a) and of LRDs (b) in the three regions.

expressed, with a time-dependent increase in their amplitude and duration, probably associated with a kind of maturation *in vitro* (Fig. 7C; $n = 4$). At the end of this 24-h period, ILEs were still completely blocked by restoring physiological concentration of Mg^{2+} (Fig. 7C), indicating the absence of any Mg^{2+}-independent type of epileptiform activity. P5 rat CHFs were also subjected to anoxic-aglycemic episodes (5 min) applied either before the occurrence of the first ILEs (Fig. 7D, a; $n = 4$) or after its expression (Fig. 7D, b; $n = 4$). In both cases, LRDs were not induced by anoxia and only repetitive ILEs were observed (Fig. 7D).

Transformation of ILEs into LRDs

LRDs were not expressed before P7 even when maintained for long duration in low-Mg^{2+} ACSF (see above) but were very frequently observed at P7 and always after P7, suggesting that they resulted from a developmental process. We then tested whether ILEs were required for the expression of LRDs. Intact P7–8 CHF preparations were superfused for long duration with low-Mg^{2+} ACSF in the presence of D-APV (40 µM), which did not affect the maintenance of LRDs (see Fig. 3B). As expected, the expression of ILEs was completely prevented ($n = 6$) as long as D-APV was present (3 h) and LRDs were not expressed during this period. Upon washout of D-APV, ILEs and LRDs reappeared sequentially (Fig. 7E) and the onset of LRDs (96 ± 8 min, $n = 6$) was not significantly different from control situations without preincubation with D-APV (see Table 1). Therefore, ILEs appeared to be required for the expression of LRDs.

LRDs were generated in intact CHFs but not in cortico-hippocampal slices

In order to determine whether LRDs can be expressed in slices, we superfused P7–8 hippocampal slices with low-Mg^{2+} ACSF and recorded in the CA1 area. For this purpose, 600-µm slices from the temporal region were used because hyperactivity is more likely to be expressed in thicker slices (Anderson *et al.*, 1986) and our preliminary results suggested that the temporal part was more likely to generate seizures than the septal part. ILEs were generated in 16 of 23 slices and had nearly the same aspect as in intact tissues. Compared to intact CHFs, there was no significant difference in the mean value for the onset of the first ILE (16.5 ± 3.5 min, $n = 16$, Fig. 8A) but its amplitude (418 ± 46 µV) and duration (53 ± 5 s) were significantly lower. During the first hour, ILEs occurred at ≈ 6/h but their frequency progressively increased to 9/h (384 ± 31 s) after

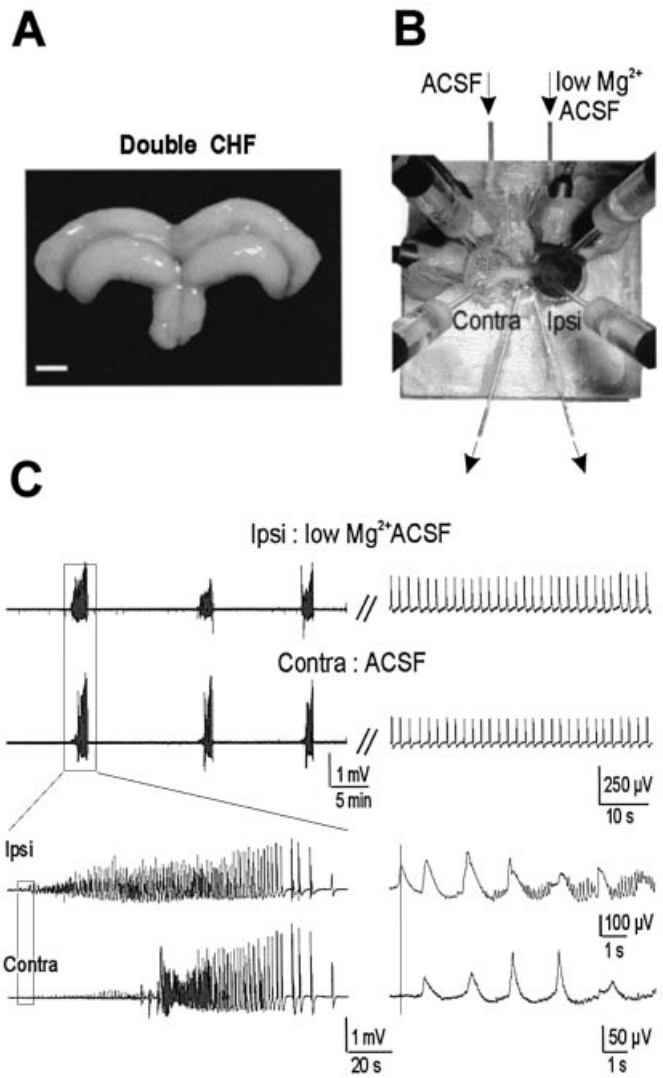

FIG. 5. Inter-hemispheric propagation of ILEs and LRDs in a P8 double CHF. (A) The photo shows the double CHF in which the connections between the two hippocampi and between the hippocampus and cortical areas have been preserved. The bar corresponds to 2.5 mm. (B) The two compartment chambers with a double CHF and four extracellular recording electrodes placed in septal and temporal CA1 areas. The ipsilateral compartment (ipsi) was superfused with low-Mg^{2+} ACSF whereas the contralateral compartment (contra) was superfused with normal ACSF. The dye (methylene blue) in the ipsilateral compartment was added to verify that the low-Mg^{2+} ACSF did not diffuse into the contralateral compartment. (C) Seizures induced by low-Mg^{2+} ACSF in the ipsilateral compartment propagated to the contralateral one without attenuation. The first ILE and LRDs recorded in each of the two compartments are represented. Note the delay between the onset of tonic phases of the first ILE recorded in temporal parts of the hippocampus but the synchronization of the clonic phases.

6 h. This increase in frequency was accompanied by a reduction in duration from 53 ± 5 to 33 ± 4 s but ILEs never gave rise to long-lasting ILEs or LRDs even after 8 h (Fig. 8A).

However, if LRDs were first generated in intact P7–8 CHFs (n = 4), they were maintained in hippocampal slices prepared from of these tissues. Indeed, LRDs recorded in the CA1 area of P7–8 intact CHFs were recovered in 600-µm slices (n = 8) prepared in low-Mg^{2+} ACSF from the temporal part of the hippocampus of these intact CHFs. Their amplitude (–15 ± 4%) and frequency (–38 ± 7%) were however, reduced (Fig. 8B). Therefore, the induction of LRDs required an intact preparation but, once generated, LRDs could be observed in slices.

Discussion

Removing the magnesium block of NMDA receptors in immature intact cortico-hippocampal formations generated two sequential types of spontaneous recurrent hyperactivity (ILEs and LRDs) which occur at different critical periods of brain development. Repetitive episodes of ILEs gave rise to LRDs that persisted for long periods in physiological conditions, suggesting that this preparation may represent a new *in vitro* model of epilepsy in immature tissues. These events are not readily seen in slices, reflecting the importance of intact preparations to study network-driven activities in neurons that possess few functional synapses.

An age-dependent transforming pattern of hyperactivity

Continuous superfusion of the immature intact cortico-hippocampal formations (CHFs) with low-Mg^{2+} ACSF resulted in two successive phases of hyperactivity: ictal-like seizures (ILEs) which then gave rise to long-lasting interictal late recurrent discharges (LRDs). These two phases had different developmental expression and pharmacological properties. Recurrent spontaneous and evoked ILEs were observed during the first postnatal week and started as early as P1. Their amplitude and duration progressively increased, indicating an age-related maturation process. As expected, this initial pattern of activity, which was prevented or completely and reversibly blocked by D-APV, was controlled by the activation of NMDA receptors. Therefore, even at early developmental stages when functional NMDA-receptor-mediated synapses have just become functional (Tyzio *et al*., 1999), the release of the magnesium block is sufficient to trigger an epileptiform activity (Traub *et al*., 1994). At the end of the first postnatal week, ILEs were progressively replaced by LRDs that were no longer sensitive to D-APV. The mechanism of this transition is not known but the induction of LRDs was also an age-dependent process which occurred around P7 and required the expression of ILEs in CHFs.

LRDs were not the consequence of anoxia in intact CHFs

LRDs have not been reported in neonatal and adult hippocampal slices superfused with low-Mg^{2+} ACSF. Although tissues from neonates are well known to be more resistant to anoxic episodes than are adult ones (Cherubini *et al*., 1989), intact CHFs could be more sensitive to anoxia than slices. However, several observations clearly suggest that LRDs could not result from accidental experimental anoxia: (i) LRDs were not expressed in P7–10 intact tissues superfused for several hours in normal ACSF or following anoxic episodes (Dzhala *et al*., 2001); (ii) in P5 CHFs superfused with low-Mg^{2+} ACSF, LRDs were never observed even after 24 h of continuous superfusion with low-Mg^{2+} ACSF or following experimental anoxic–aglycemic episodes; (iii) preventing anoxia during the preparation of P8 CHFs, using a low concentration of Ca^{2+} or by blocking NMDA receptors (Krnjevic, 1990; Papas *et al*., 1993), did not block the expression of LRDs in low-Mg^{2+} ACSF; (iv) in P7–8 intact CHFs superfused with low-Mg^{2+} ACSF the expression of LRDs was either prevented or significantly delayed following an anoxic-aglycemic episode. Furthermore, once expressed, LRDs were inhibited by anoxia. Therefore, LRDs were not the consequence of anoxia even when anoxia was experimentally induced.

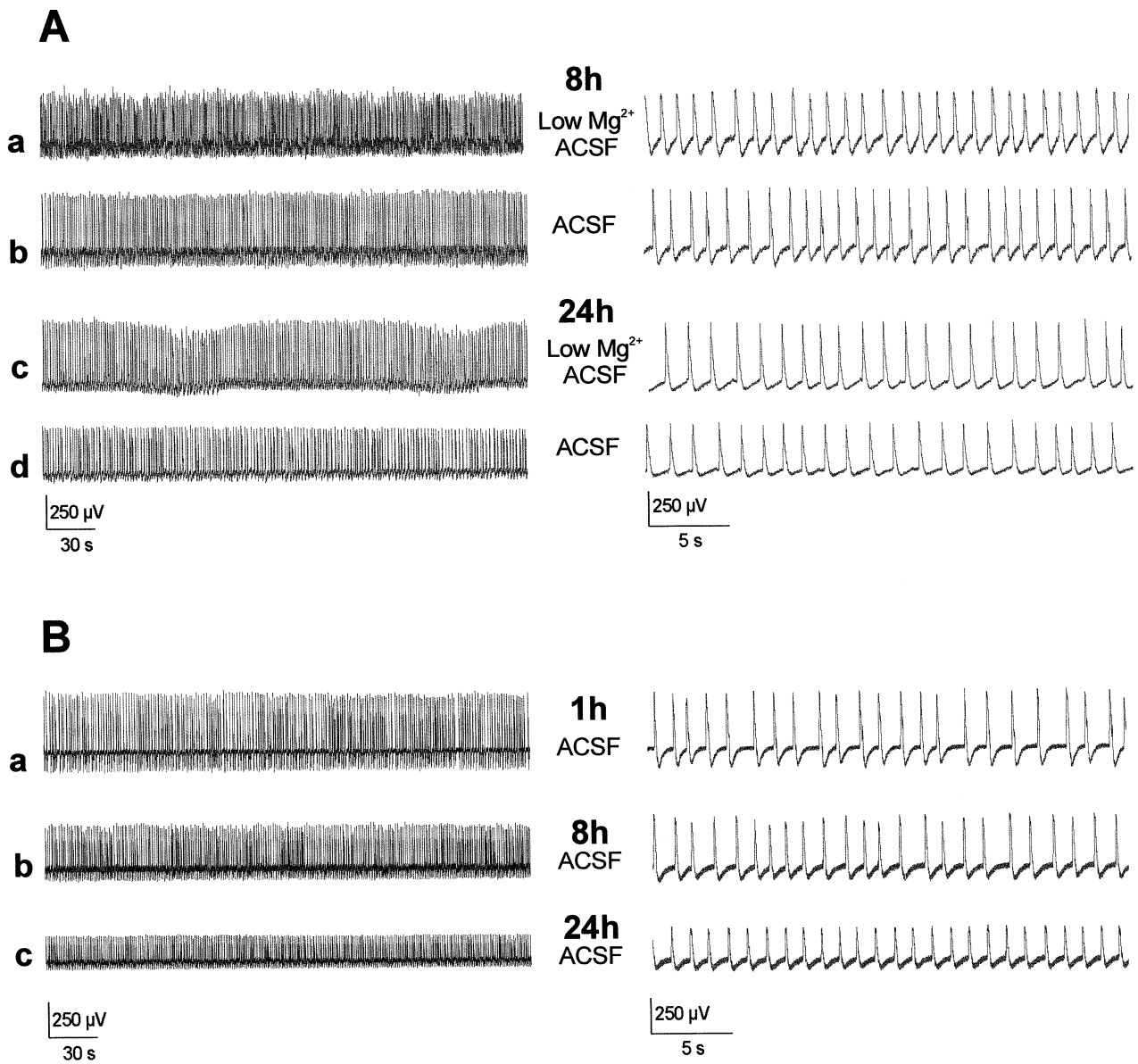

FIG. 6. Long-lasting recordings of LRDs induced in P7–8 CHFs. (A) Persistent LRDs in low-Mg^{2+} ACSF: (a) LRDs were first generated in low-Mg^{2+} ACSF and recorded 6–8 h later in the CA1 area. (b) CHFs were then washed for 1 h with normal ACSF and recorded in ACSF. Tissues were then superfused with low-Mg^{2+} ACSF for 30 min and placed in 250 mL of well-oxygenated low-Mg^{2+} ACSF at 30 °C. (c) 16–18 h after, tissues were placed in the perfusion chamber and LRDs were recorded in the CA1 area for at least 30 min in low-Mg^{2+} ACSF and then (d) in normal ACSF after equilibration for 1 h. (B) Persistent LRDs in ACSF: intact CHFs were superfused with low-Mg^{2+} ACSF until the expression of LRDs in the CA1 area. After 1–2 h the superfusing buffer was changed for ACSF and records were performed after 1 h (a) and 6–8 h (b) in the same medium. Tissues were placed overnight in 250 mL of well-oxygenated ACSF at 30 °C and (c) LRDs were recorded in the CA1 area 24 h after the initial application of ACSF.

A specific model of intact tissues

Already at P1, ILEs were consistently recorded in intact CHFs superfused with the low-Mg^{2+} ACSF. In contrast, in hippocampal slices, interictal activities (Kohling *et al.*, 2000; Weissinger *et al.*, 2000) are mainly generated during the first postnatal week whereas ILEs are more consistently generated during the second postnatal week in cortical and hippocampal slices (Hegstad *et al.*, 1989; Gloveli *et al.*, 1995; Wang & Jensen, 1996; Sabau *et al.*, 1999; Wong & Yamada, 2001). ILEs have also been observed in the intact hippocampus using different convulsive agents (Khalilov *et al.*, 1997b; Khalilov *et al.*, 1999; Luhmann *et al.*, 2000) but again not in age-matched slices, further confirming the relevance of intact tissues for studying early epileptiform processes. Clearly, the minimal density of synapses required to trigger synchronization of the neuronal population (Jefferys, 1998) and to an ictal-like discharge is achieved earlier in the intact structure than in conventional slices.

Furthermore, in intact P7–8 CHFs maintained in low-Mg^{2+} ACSF, ILEs evolve with age and are transformed into LRDs. We have not observed a similar transformation in P7–8 hippocampal slices. Furthermore, this evolution of ILEs towards LRDs does not occur in intact hippocampal preparations where ictal-like events have been induced for several hours with bicuculline (Khalilov *et al.*, 1997b), 4-aminopyridine (Luhmann *et al.*, 2000) or kainic acid (Khalilov *et al.*, 1999). This difference suggests that specific activation of NMDA receptors may be required to initiate such a pattern of

activity, as the other convulsive agents have no direct effect on these excitatory receptors. Therefore, the evolution of ILEs towards LRDs appeared to be a characteristic of seizures induced by low-Mg^{2+} in intact tissues. This preparation is also more sensitive than slices to persistent activation and therefore to plastic modifications induced by seizures that can occur at early developmental stages.

The site of initiation of seizures also appears to be different in slices and intact tissues. In immature cortico-hippocampal slices, ILEs are found to be generated more frequently in entorhinal cortex and neocortical regions than in the hippocampal formation (Weissinger *et al*., 2000). In contrast, in intact CHFs, ILEs occur simultaneously in cortical and hippocampal areas and can be generated in the hippocampus proper disconnected from cortical afferents. Furthermore, seizures also appeared with nearly no delay in CA1 and CA3 regions and in the dentate gyrus, excluding a particular pacemaker population of neurons as reported in slices (Weissinger *et al*., 2000).

A specific model of immature tissues

The pattern of discharges induced by low-Mg^{2+} ACSF in intact neonatal structures displays some similarities with that generated in adult cortico-hippocampal slices where ILEs followed by LRDs have been observed in cortical areas but not in the hippocampus (Heinemann *et al*., 1993). However, glutamate and GABA receptors are involved differently in seizures induced in mature and immature tissues. The main difference lies in the fact that LRDs are insensitive to NMDA receptor antagonists and to physiological concentrations of magnesium ions in intact CHFs, whereas LRDs observed in adult cortical slices are blocked by D-APV and do not persist in physiological ACSF (Zhang *et al*., 1994). The mechanisms of these differences are not known. Furthermore, the $GABA_A$ receptor-mediated inhibition was still functional in slices (Tancredi *et al*., 1988; Benardo, 1993; Whittington *et al*., 1995). In contrast, high concentrations of isoguvacine are required to prevent ILEs in CHFs, indicating either a loss of the efficacy of GABAergic synapses (Pfeiffer *et al*., 1996) or a modification of the properties of $GABA_A$ receptors. The entry of calcium ions through NMDA receptors known

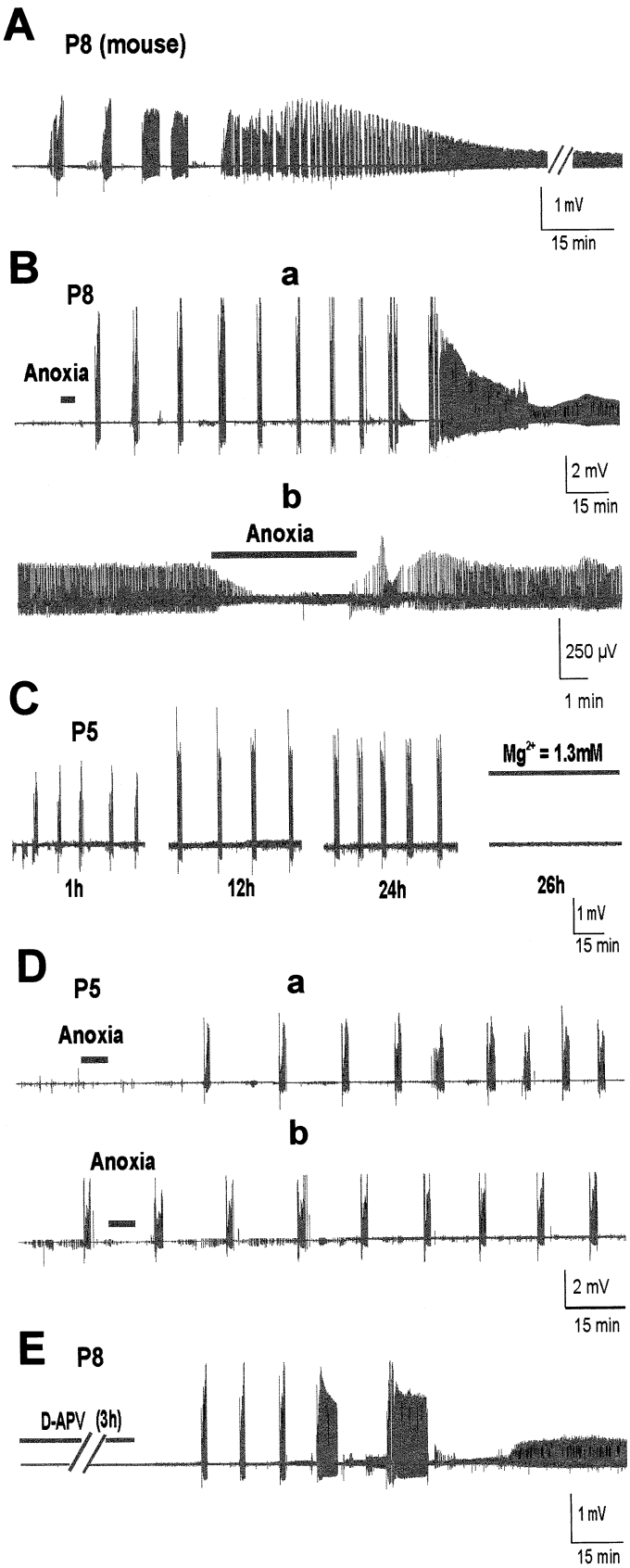

FIG. 7. Characteristics of LRDs. Trace A was from a mouse and traces B–E were all from rats. (A) Low-Mg^{2+}-ACSF-induced seizures (ILEs and LRDs) in the CA1 area of P8 mice. ILEs and LRDs were sequentially expressed in mice CHFs, which are much smaller than those of P8 rats. (B) Anoxia did not facilitate the expression of LRDs. (Ba) An anoxic–aglycemic episode of 5 min (bar) was performed on CHFs from P7 rats superfused with low-Mg^{2+} ACSF. Upon reoxygenation, ILEs and LRDs were sequentially induced in the CA1 area. Note that LRDs were not immediately expressed following the anoxic episode, and also the increase in the number of ILEs expressed in the CA1 area compared to the situation without anoxia. (Bb) Stable LRDs were first generated in CHFs of P7 rats superfused with low-Mg^{2+} ACSF. An anoxic–aglycemic episode of 5 min (bar) was then performed; it rapidly suppressed LRDs. They were restored upon reoxygenation. (C) LRDs were not expressed in P5 CHFs. Tissues were continuously superfused with low-Mg^{2+} ACSF. Records in the CA1 area were performed after 1, 12 and 24 h in this medium and after restoring the physiological concentration of magnesium ions. Note the absence of LRDs in low-Mg^{2+} ACSF and the blockade of ILEs by magnesium ions after 26 h. (D) Anoxia did not generate LRDs in P5 CHFs. Tissues were superfused with low-Mg^{2+} ACSF and an anoxic–aglycemic episode of 5 min (bar) was performed either (a) before the occurrence of the fist ILE or (b) after the second ILE. In both cases no LRDs were generated. (E) ILEs are required in P7–8 CHFs for the expression of LRDs: tissues were superfused with low-Mg^{2+} ACSF containing D-APV (40 µM) for 3 h (bar) and then washed with the same medium without D-APV. Note that no ILEs or LRDs were induced during D-APV application. They were, however, sequentially expressed when D-APV was removed.

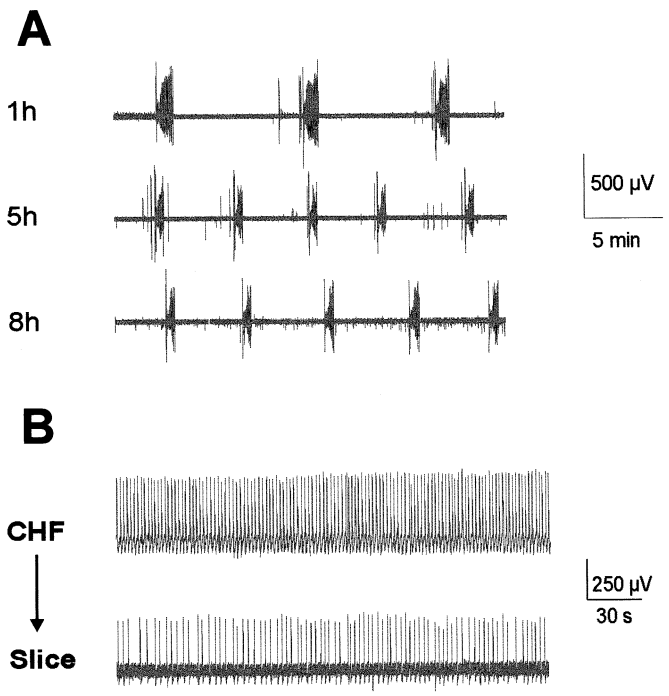

FIG. 8. LRDs were not expressed in slices: (A) P8 slices (600 µm) were continuously superfused with low-Mg^{2+} ACSF. ILEs were repetitively recorded in the CA1 area over 8 h but no LRDs were expressed. Note that the duration of ILEs progressively reduced. (B) First, LRDs were induced in the CA1 area of P8 intact CHFs over a 1-h period. Tissues were then transferred to low-Mg^{2+} ACSF and sliced. Temporal slices (600 µm) were placed in the perfusing chamber and superfused with low-Mg^{2+} ACSF and LRDs were recorded in the CA1 area.

to activate calcium-dependent kinases that phosphorylate $GABA_A$ receptors, leading to their apparent desensitization (Stelzer *et al*., 1987), may also be involved.

Synchronized seizures were found in hippocampal and cortical areas in immature intact tissues whereas, in adult cortico-hippocampal slices, ILEs are initiated in cortical areas (Dreier & Heinemann, 1991) but not in the hippocampus (Mody *et al*., 1987; Kohling *et al*., 1994; Gloveli *et al*., 1995) where only short recurrent discharges, distinct from LRDs, have been consistently recorded (Heinemann *et al*., 1993). This difference between cortical and hippocampal areas has been explained by the filtering role played by the dentate gyrus in preventing or attenuating the entry into the hippocampus of any hyperactivity (Dreier & Heinemann, 1991; Jones, 1993). In intact CHFs, a less efficient control of this entry during the early developmental stage is consistent with the propagation of ILEs from cortical regions to the intact hippocampus. However, the delay of propagation of ILEs between cortical and hippocampal regions, which is limited to few milliseconds, rather suggests that, in contrast to adult slices, ILEs are directly expressed in the hippocampus and indeed ILEs have been recorded in the intact hippocampus disconnected from cortical areas. This may be related to different subunit composition of NMDA receptors between the adult and developing hippocampus (Monyer *et al*., 1994; Khazipov *et al*., 1995; Wenzel *et al*., 1997; Kirson *et al*., 1999) or to a higher density of recurrent fibres that are transiently expressed during brain development, as reported recently at birth for CA1 axons (Aniksztejn *et al*., 2001).

A persistent pattern of epileptiform activity

The late recurrent discharges generated in P7–8 CHFs persist at least 24 h in the presence of low-Mg^{2+} ACSF. They were not abolished by D-APV or by restoring physiological concentrations of Mg^{2+} ions, suggesting that superfusion with low Mg^{2+} in immature intact structures represents a model of epilepsy and not a model of acute seizures. The persistence of this pattern does not result from the long-duration superfusion with low-Mg^{2+} because, once generated, restoration of physiological concentration of Mg^{2+} does not suppress this late recurrent activity, at least within 24 h. The observation that bicuculline triggers the expression of LRDs that persist after washout of the drug is also consistent with the fact that LRDs are usually persistent after initiation.

Furthermore, this epileptiform activity was also maintained in slices prepared from intact tissues in which LRDs had been initially induced, suggesting that intact tissues are required for the induction of LRDs but that their expression can also occur in slices. Interestingly, LRDs are also able to propagate over long distances in physiological conditions. This is illustrated by the experiments using the double CHF preparation in which right and left CHFs were maintained interconnected through the commissure. LRDs generated in one CHF propagate to the second CHF that has been maintained in physiological conditions. This model of hyperactivity and particularly the preparation using the two interconnected CHFs may be particularly useful in the development of antiepileptic drugs. It offers the advantage over other *in vitro* preparations of allowing the study of an epileptic tissue that has never been in contact with the agent responsible for the hyperactivity.

Conclusion

A new model of long-lasting hyperactivity has been obtained in intact CHFs from immature rats and mice by initial activation of NMDA receptors during a crucial period of brain development. The characteristics of this model are specific for immature rats, different from the slice model and distinct from other immature models in intact tissues. They suggest that this new model may provide a useful tool for studying *in vitro* some aspects of human infantile epilepsies.

Acknowledgements

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Abbreviations

ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APV, 2-amino-5-phosphono-pentanoic acid; CA, cornu ammonis; CHF, cortico-hippocampal formation; CNQX, 6-cyano-7-nitro quinoxaline-2,3-dione; E, embryonic day; GABA, gamma-aminobutyric acid; GYKI 52466, 1-[4-aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine; ILE, ictal-like event; KA, kainic acid; LRD, late recurrent discharge; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7-sulphonamide; NMDA, *N*-methyl-D-aspartate; P, postnatal day.

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