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Layer-Specific Generation and Propagation of Seizures in Slices of Developing Neocortex: Role of Excitatory GABAergic Synapses

Sylvain Rheims, Alfonso Represa, Yehezkel Ben-Ari, and Yuri Zilberter

Institut de Neurobiologie de la Mediterranee, Institut National de la Santé et de la Recherche Médicale U901, Université de la Méditerranée, Marseille, France

Rheims S, Represa A, Ben-Ari Y, Zilberter Y. Layer-specific generation and propagation of seizures in slices of developing neocortex: role of excitatory GABAergic synapses. *J Neurophysiol* 100: 620–628, 2008. First published May 21, 2008; doi10.1152/jn.90403.2008. The neonatal period is critical for seizure susceptibility, and neocortical networks are central in infantile epilepsies. We report that application of 4-aminopyridine (4-AP) to immature (P6–P9) neocortical slices generates layer-specific interictal seizures (IISs) that transform after recurrent seizures to ictal seizures (ISs). During IISs, cell-attached recordings show action potentials in interneurons and pyramidal cells in L5/6 and interneurons but not pyramidal neurons in L2/3. However, L2/3 pyramidal neurons also fire during ISs. Using single *N*-methyl-D-aspartate (NMDA) channel recordings for measuring the cell resting potential (E_m), we show that transition from IISs to ISs is associated with a gradual E_m depolarization of L2/3 and L5/6 pyramidal neurons that enhances their excitability. Bumetanide, a NKCC1 co-transporter antagonist, inhibits generation of IISs and prevents their transformation to ISs, indicating the role excitatory GABA in epilepsies. Therefore deep layer neurons are more susceptible to seizures than superficial ones. The initiating phase of seizures is characterized by IISs generated in L5/6 and supported by activation of both L5/6 interneurons and pyramidal cells. IISs propagate to L2/3 via activation of L2/3 interneurons but not pyramidal cells, which are mostly quiescent at this phase. In superficial layers, a persistent increase in excitability of pyramidal neurons caused by E_m depolarization is associated with a transition from largely confined GABAergic IIS to ictal events that entrain the entire neocortex.

INTRODUCTION

The neonatal period is critical for seizure susceptibility. The incidence of seizures is indeed higher in neonates than in older children or adults (Mizrahi 1999). Neonates with seizures are at risk for death, and survivors are at risk for neurological impairment, developmental delay, and later epilepsy (Bye et al. 1997; Dixon et al. 2002; Rennie and Boylan 2003; Scher et al. 1993; Tekgul et al. 2006). Several observations suggest that a high incidence of seizures during neonatal period may be caused by intrinsic properties of the neonatal brain. For instance, 10–25% of infants present normal examination and normal MRI (Lombroso 1996; Scher et al. 1993; Tekgul et al. 2006; Tharp 2002) despite the high prevalence of cerebral lesions (Bye et al. 1997; Lombroso 1996; Rennie and Boylan 2003; Scher et al. 1993; Tekgul et al. 2006; Tharp 2002). Many infants, including those suffering from cerebral lesions, experience a seizure-free period of months to years, suggesting that early seizures may be self-limited possibly because of the developmental alterations in neuronal excitability (Tharp 2002).

Although neocortical networks are central in infantile epilepsies (Mizrahi 1999; Mizrahi and Clancy 2000), our understanding of epilepsies in the developing brain comes from studies on the immature hippocampus (for review, Ben-Ari 2002; Ben-Ari et al. 2004; Cossart et al. 2005; Le Van Quyen et al. 2006; Lienekugel et al. 1999). GABA excites immature neurons in the hippocampus (for review, Ben-Ari 2002; Ben-Ari et al. 2007) and neocortex (Owens et al. 1996, Rheims et al. 2008; Yamada et al. 2004), but while this action directly impacts seizures in hippocampus (Dzhala and Staley 2003; Dzhala et al. 2005; Khalilov et al. 1999, 2005; Khazipov et al. 2004; Lienekugel et al. 1997), there is no evidence that this is the case for neocortical neurons.

Here we explore the mechanisms of initiation and development of paroxysmal oscillations in deep and superficial neocortical layers (L2/3 and L5/6) of neonatal mice. We report that 4-aminopyridine (4-AP) triggers seizures that initially are interictal (IIS), generated by L5/6 neurons. Interictal events propagate to L2/3 via activation of L2/3 interneurons but not pyramidal cells. With the recurrence of seizures, there is a transition from interictal to ictal events (ISs), which are maintained by activity of interneurons and pyramidal cells in both deep and superficial layers. This transition is associated with a gradual depolarization of E_m in both L2/3 and L5/6 pyramidal neurons. Therefore our results show that the layer specific excitatory action of GABA plays an important role in the generation of seizures. In addition, recurrent seizures induce a persistent increase in neuron excitability that underlies the transition from interictal to ictal paroxysmal activity entraining the entire cortical network.

METHODS

Slices preparation

Brain slices were prepared from postnatal day (P)6 to P17 Swiss mice of both sexes. P0 was the day of birth. All animal protocols conformed to the French Public Health Service policy and the INSERM guidelines on the use of laboratory animals. Animals were decapitated, and brains were removed. Saggital slices (300–400 μm) were cut by a Microm slicer (International) using ice-cold oxygenated modified artificial cerebrospinal fluid (mACSF) containing 0.5 mM $CaCl_2$ and 7 mM $MgSO_4$, and with Na^+ replaced by an equimolar concentration of choline. Slices were transferred to a normal ACSF containing (in mM) 126 NaCl, 3.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$,

1.3 MgCl$_2$, 2.0 CaCl$_2$, and 10 D-glucose, pH 7.4, at room temperature (20–22°C) for $\geq$1 h before use.

Electrophysiology

For recordings, slices were placed into a conventional fully submerged chamber superfused with ACSF (32–34°C) at a rate of 2–3 ml/min. Cell types were identified by IR-DIC video microscopy. Patch-clamp recordings were performed using dual EPC-9 or EPC-10 amplifiers (HEKA Elektronik). Pipettes (resistance of 3.5– 8 MOhm) were pulled from borosilicate glass capillaries. Patch-clamp recordings in the cell-attached configuration were performed using the following pipette solutions (in mM): *1*) for single GABA channels, 140 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 0.01 GABA, pH adjusted to 7.3 by NaOH; and *2*) for single *N*-methyl-D-aspartate (NMDA) channels, 140 NaCl, 2.5 KCl, 2 CaCl$_2$, 10 HEPES, 0.01 NMDA, and 0.01 glycine, pH adjusted to 7.3 by NaOH. Analysis of currents through single channels and current-voltage relationships were performed using Clampfit 9.2 (Axon Instruments, Union City, CA) as described previously (Tyzio et al. 2003, 2006). Patch-clamp recordings in the whole cell configuration were performed using pipette solution containing (in mM) 115 potassium gluconate, 20 KCl, 4 ATP_Mg, 10 Na_phosphocreatine, 0.3 GTP_Na, 10 HEPES, and biocytin 0.5%, pH 7.3 adjusted by NaOH. Similar solution has been used for recordings of action potentials.

Field potentials were recorded using electrodes made from borosilicate glass capillaries filled with ACSF. Signals were amplified using DAM8A (World Precision Instruments).

Continuous recordings were digitized (10 kHz) on-line using a Digidata 1322 (Axon Instruments) and analyzed off-line with Clampfit 9.0 (Axon Instruments). Noncontinuous recordings were digitized (10 or 50 KHz) on-line and analyzed off-line (Igor Wavemetrics, Lake Oswego, OR).

Pharmacology

Drugs were purchased from Tocris (gabazine, NBQX, D-APV, 1,3-dipropyl-8-cyclopentylxanthine, Carbachol), Sigma (4-AP, biocytin, picrotoxin, bumetanide), and Roche Pharmaceutical Division (diazepam).

Experimental model

Among a variety of experimental models for seizure induction, we selected the 4-action potential (AP) model for the following reasons: *1*) a relatively low concentration of 4-AP (50 μM) readily induced seizure activity in neonatal neocortex; *2*) 4-AP did not affect the neurotransmitter receptors involved in seizure generation; and *3*) 4-AP increased the level of synaptic activity and thus the entire excitability of neuronal network. The last effect can be explained by widening of APs caused by a partial block of K$^+$ channels that results in a bigger Ca^{2+} influx into presynaptic terminals and thus higher neurotransmitter release probability (Zilberter, unpublished data).

Histological processing

To show biocytin-injected cells, slices were immersed in a fixative solution of paraformaldehyde (4%) and glutaraldehyde (0.2%) overnight at 4°C after electrophysiological recording. To increase penetration of the reagents used for biocytin detection, slices were quickly frozen on dry ice and thawed in phosphate buffer. Slices were rinsed in 0.05 M Tris-buffered saline (TBS), pH 7.4, containing 0.3% Triton X-100 for 30 min and incubated overnight at 4°C in an avidin–biotin–peroxidase solution prepared in TBS according to the manufacturer's recommendation (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA). After a 30-min wash in TBS and a 10-min rinse in Tris buffer (TB), pH 7.6, slices were processed for 15 min in 0.06%

3-3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide diluted in TB. The slices were rinsed in TB for 30 min, mounted on gelatin-coated slides, dehydrated, and coverslipped with permount. Stained cells were reconstructed using a camera lucida. Axonal size and number of branching nodes were analyzed using the analysis software National Institutes of Health ImageJ 1.33d (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Random stimulation of nerve fibers

Nerve fibers were stimulated 3–5 times each for 30 s via a bipolar metal extracellular electrode (50 μm) positioned either in L1 or white matter. Each stimulus consisted of a random sequence of 0.2-ms pulses (minimal interpulse interval, 20 ms) for 500 ms.

Statistical analysis

Group measures are expressed as mean $\pm$ SE; error bars also indicate SE. The statistical significance of differences was assessed with the Student's *t*-test. The level of significance was set at $P < 0.05$.

RESULTS

Neocortical seizures are initiated in L5/6

About 3 min after application of 4-AP (50 μM), we observed an increase in frequency of cortical giant depolarizing potentials (cGDP) occurrence in L5/6 (Fig. 1): 3.7 $\pm$ 0.8/min in control and 17.8 $\pm$ 3.6/min in the presence of 4-AP ($n =$ 18). In L2/3, field recordings showed the initial episodes of paroxysmal activity (IISs) 7 $\pm$ 3 min following application of 4-AP (Fig. 1). IISs in L2/3 occurred at a frequency of 2.6 $\pm$ 0.8/min, always synchronously with those simultaneously recorded in L5/6 ($n = 16$). By contrast, some IISs in L5/6 were not associated with oscillations in L2/3, suggesting that they were generated in deep layers and propagated to superficial ones (Fig. 1, *inset b*). Analysis of a delay between synchronous L2/3 and L5/6 IIS confirmed this suggestion: L5/6 oscillations always preceded those in L2/3, with a mean delay of 97.8 $\pm$ 8.5 ms (Fig. 1). After exposures to 4-AP exceeding 19 $\pm$ 7 min, an additional pattern of IS appeared (Fig. 1). These events consisted of several biphasic potentials (20 $\pm$ 5-s duration, 1 event per 10 min; Fig. 1, *inset c*). ISs occurred simultaneously in all layers. Oscillations in L2/3 preceded sometimes those in L5/6, and the delay between L5/6 and L2/3 ictal-like events was 33.2 $\pm$ 10.2 ms. Therefore the process of seizure generation can be roughly divided by two phases: *1*) an initiating phase characterized by interictal seizures generated in L5/6 and propagating to L2/3 and *2*) an ictal seizure phase manifested by oscillations of the entire neocortical network.

Interneurons trigger seizures in L2/3

To identify the neuronal subtypes preferentially involved in the IIS pattern generation, we recorded (cell-attached) interneuron-pyramidal cell pairs in combination with field recordings in both L2/3 and L5/6 (Fig. 2). In L5/6, both interneurons and pyramidal cells showed the AP activity in control associated with bursts of firing corresponding to cGDPs (Fig. 2*B*). Both cell types were involved in generation of IISs (Fig. 2B, $n = 6$). In contrast, shortly after application of 4-AP, some L2/3 interneurons showed high activity correlated with L2/3 IIS (Figs. 2*A* and 3*A*, $n = 27$), whereas pyramidal cells remained mostly quiescent ($n = 11$). They remained quiescent
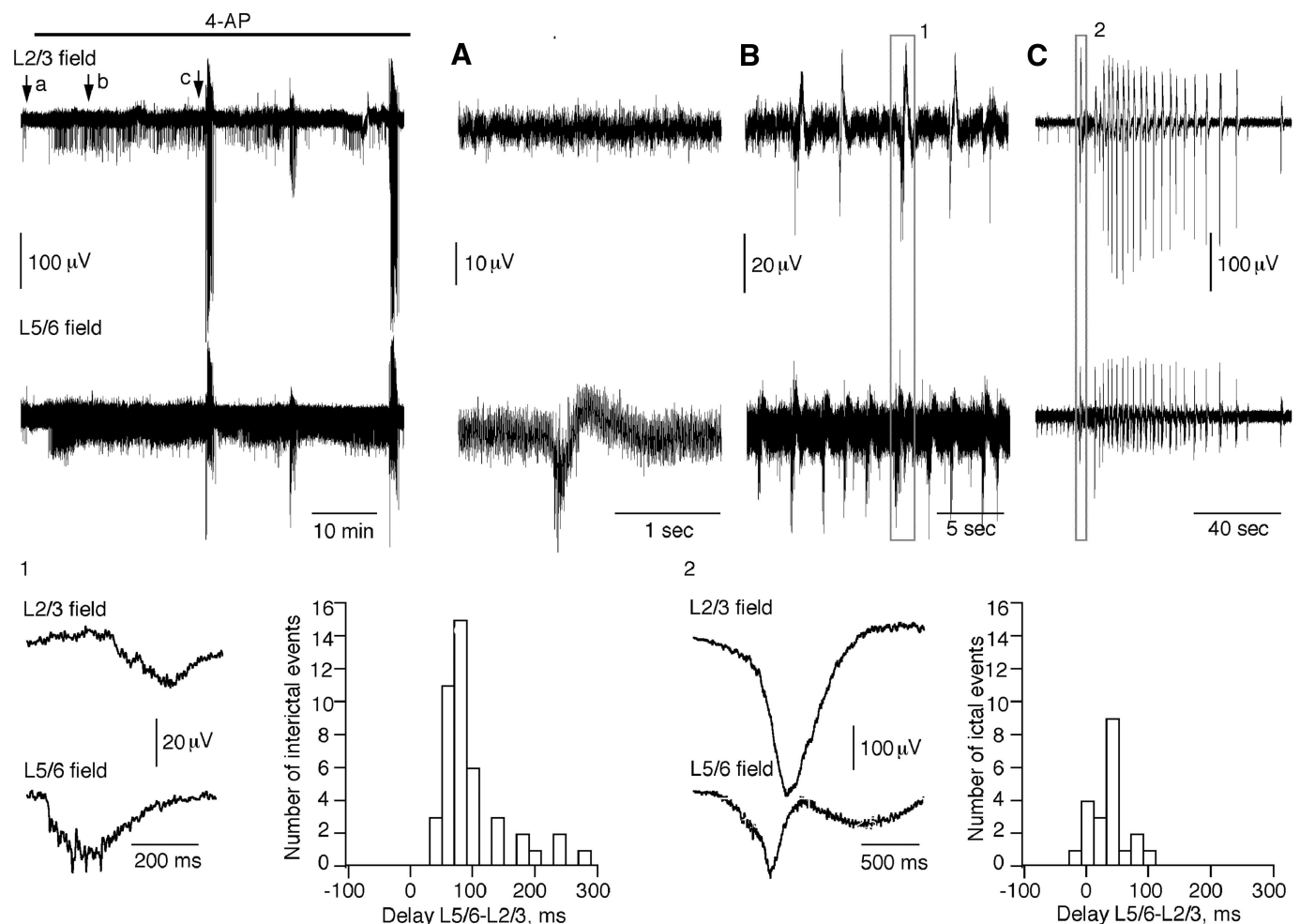
FIG. 1. The process of seizure generation can be roughly divided by 2 phases: the interictal phase and the ictal phase. Simultaneous field recordings from L2/3 and L5/6. In control, cortical giant depolarizing potentials (cGDP) occur in L5/6, whereas L2/3 is typically silent (*A*). At the interictal phase, oscillations are initiated in L5/6 and propagate to L2/3 with a mean delay of 97.8 ms (*B* and *1*). At the ictal phase, events consisted of several biphasic potentials of a total 20 ± 5 s duration and occurred at a frequency of about 1 event per 10 min (*C*) occurring simultaneously in L2/3 and L5/6 (*2*).

until transition to the ictal phase (Figs. 2*B* and 6*A*). These results suggest that, at the initiation phase of seizure generation, IISs in L2/3 are supported by activity of interneurons but not pyramidal cells.

However, pyramidal cells in L2/3 do receive a strong synaptic input during the initiation phase of seizure generation. We performed dual whole cell recordings from L2/3 pyramidal cells in combination with field recordings (Fig. 3*B*). One cell was artificially depolarized to about −20 mV by current injection. Paroxysmal oscillations induced large and long-lasting synchronous deviations of the membrane potential in both cells. In the pyramidal neuron held at a more negative potential, these events were depolarizing, although they were subthreshold and did not initiate APs. In the other pyramidal neuron, the events had opposite polarity indicating their GABAergic origin (the pipette solution contained 20 mM Cl⁻ corresponding to the Cl⁻ reversal potential of about −49 mV; note that this value is close to E_{GABA}; Rheims et al. 2008). Importantly, seizure activity and synchronous events in L2/3 pyramidal cells were abolished by application of a selective GABA$_A$R antagonist gabazine (10 μM). These results show that, during IIS L2/3, interneurons provide a strong depolarizing input to pyramidal cells, which, however, is insuf-

ficient for AP initiation because of a high AP threshold (Rheims et al. 2008).

The developmental profile of E_m and E_{GABA} shows that a switch in GABA action occurs after P10 (Rheims et al. 2008), suggesting that the pivotal role of excitatory GABAergic transmission in seizure initiation is transient. Indeed, in more mature animals (P15–P17), we observed that L2/3 pyramidal cells showed high activity correlated with L2/3 IIS (Supplemental Fig. 1*A*; $n = 8$).[1] In addition, seizure activity was increased following application of gabazine (1 μM; Supplemental Fig. 1*B*; $n = 3$).

GABAergic transmission plays a potent role in seizure initiation and development

In hippocampal neurons, glutamatergic excitatory postsynaptic potentials (EPSPs) are required for seizure generation by a variety of agents since antagonists of AMPARs fully prevent seizures (for review, Ben-Ari et al. 2007). In neocortex, in line with generation of IISs by activity of both L5/6 interneurons and pyramidal cells, application of APV + NBQX before 4-AP

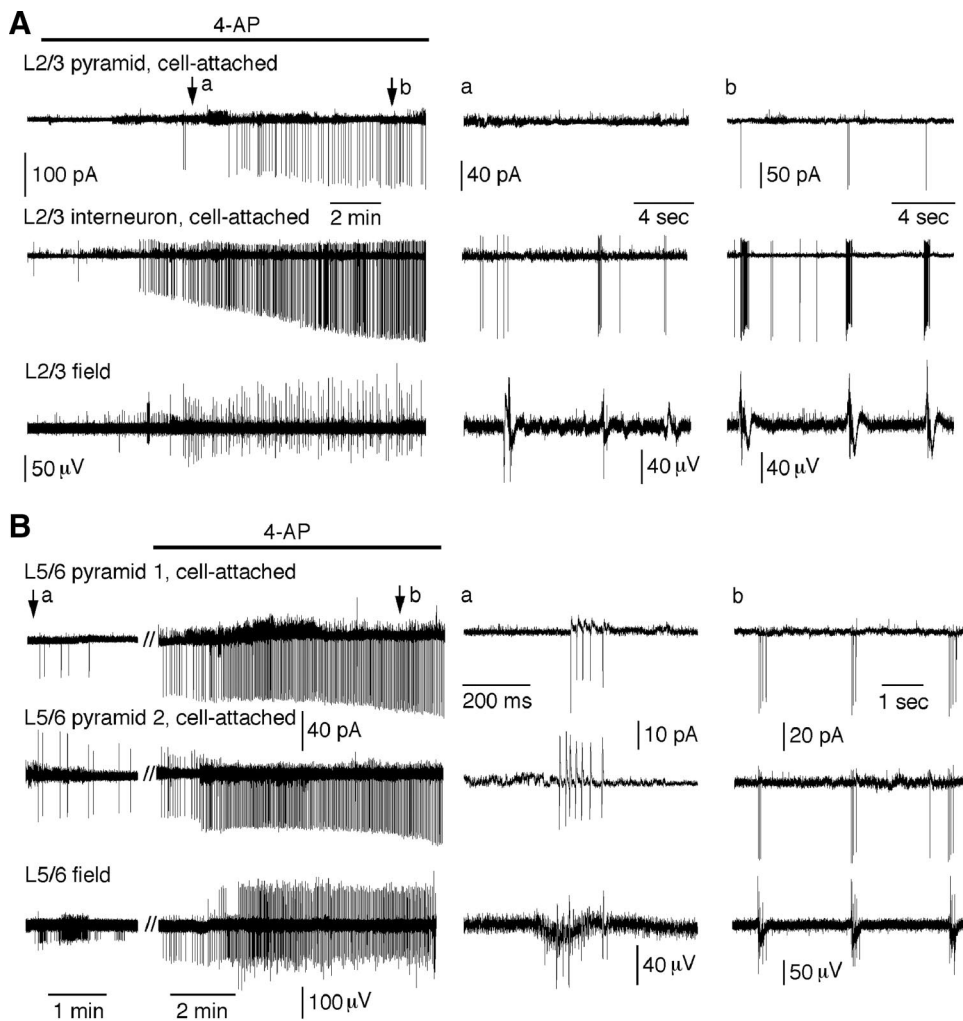[1] The online version of this article contains supplemental data.

FIG. 2. Neuronal activity during seizure initiation in L2/3 and L5/6. *A*: simultaneous cell-attached recordings from the L2/3 pyramidal cell and interneuron in combination with field recordings in L2/3. *B*: simultaneous cell-attached recordings from L5/6 pyramidal cells in combination with field recordings in L5/6.

prevented seizure induction in all experiments ($n = 7$). However, neither APV alone nor NBQX blocked seizures after their initiation ($n = 6$). Furthermore, the mixture of these antagonists terminated seizures in only one of four experiments. This suggests that glutamatergic EPSPs provide the excitatory drive for interneurons required for their synchronization during cortical seizures. Afterward, network oscillations may be supported solely by the interneuron activity.

To test this issue further, we enhanced the efficacy of GABAergic transmission by 5 μM diazepam (DZP), a benzodiazepine modulator of GABA$_A$Rs. In five of eight experiments, application of 4-AP + DZP on the background of APV + NBQX induced spontaneous IISs in L2/3 and synchronous depolarizations in whole cell recorded neurons (Fig. 4*A*). Extracellular stimulation by a single electrical shock (0.2 ms) also induced pronounced field oscillations, which were completely terminated by gabazine (Fig. 4*B*).

In addition, shifting the action of GABAergic transmission from excitatory to inhibitory may block seizure development. Bumetanide (10 μM, $n = 8$; Fig. 5*A*), which reduces [Cl$^-$]$_i$ antagonizing NKCC1, reduced seizures in L5/6 and almost completely blocked seizures in L2/3. In L5/6, the seizure frequency was 33.7 $\pm$ 17.3% of that observed in the presence of 4-AP alone ($P < 0.02$). In L2/3, bumetanide completely blocked seizures in three of four experiments (Fig. 5*A*). Im-

portantly, bumetanide prevented transition to the ictal phase: 4-AP applied on the background of bumetanide failed to induce IS despite the long-lasting (>1 h) applications. IS, however, did occur after wash out of bumetanide (Fig. 5*B*; $n = 2$).

We conclude that seizure initiation and seizure propagation from deep to superficial layers are supported by the excitatory GABAergic transmission in the neonatal neocortex.

Interictal to ictal phase transition: the role of membrane depolarization

Clearly, a seizure development to ictal-like events requires activity of the whole neuronal network including the L2/3 pyramidal one. Indeed, when seizures developed to the ictal-like mode, both interneurons and pyramidal cells showed pronounced activity synchronous with seizures recorded in all layers. Even L2/3 pyramidal cells, which were mostly quiescent during the interictal phase, were highly active at the ictal phase (Fig. 6*A*) Therefore after transition into ictal-like events, seizures were maintained by activity of pyramidal cells and interneurons in both deep and superficial layers.

As a possible mechanism of such transition, we suggested that the intense synaptic activity during the initial stage of seizure generation might result in a gradual membrane depolarization. This would facilitate seizure development by *1*) a

A

L2/3 pyramid; cell-attached

10 sec   200 pA

L2/3 interneuron; cell-attached

50 µV

L2/3 field
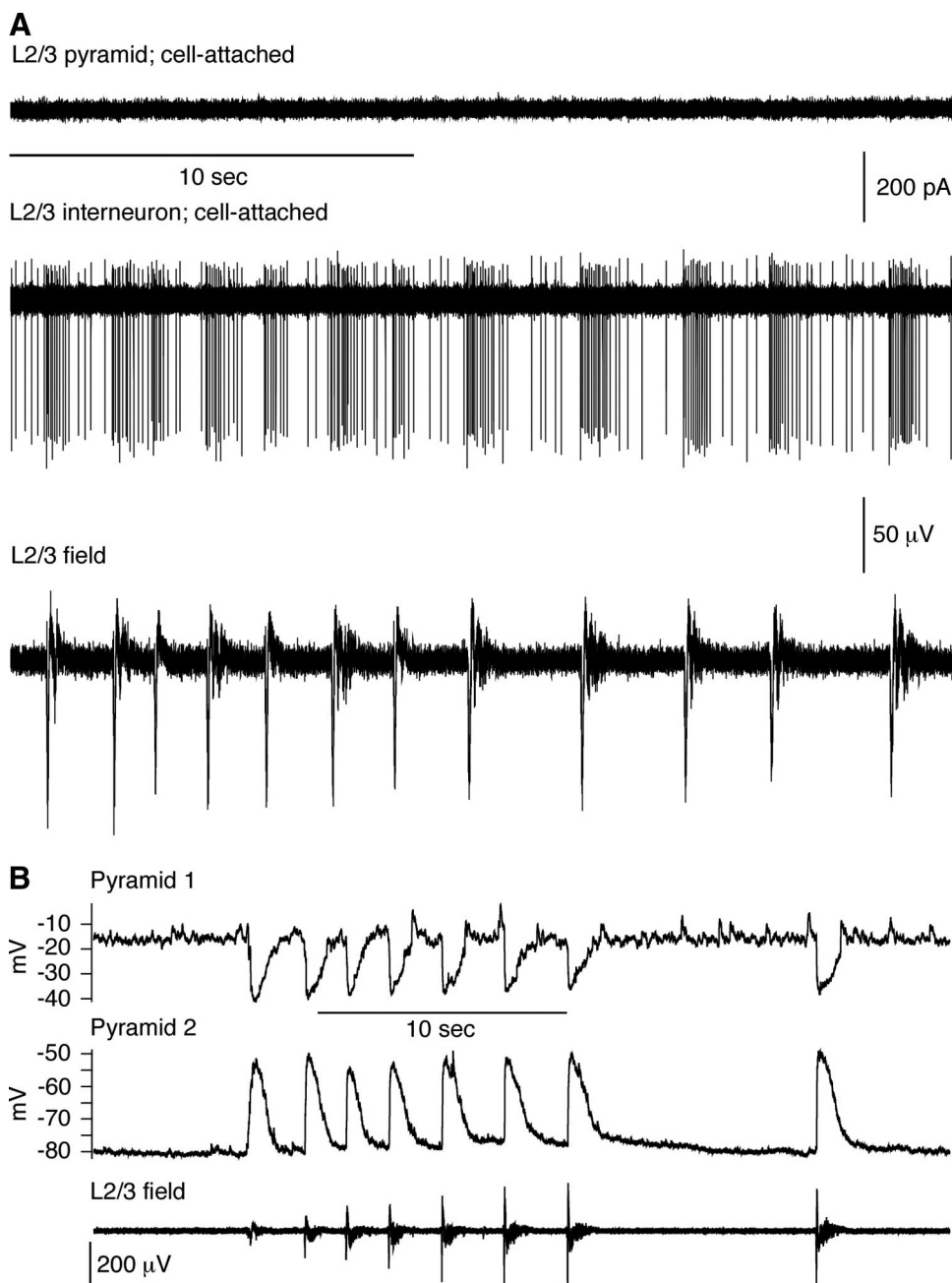
B   Pyramid 1

Pyramid 2

L2/3 field

200 µV

FIG. 3. Synaptic input to L2/3 pyramidal cells during interictal seizures is mostly GABAergic. *A*: simultaneous cell-attached recordings from the L2/3 pyramidal cell and interneuron in combination with field recordings in L2/3. *B*: simultaneous whole cell recordings from 2 L2/3 pyramids in combination with field recordings. Pyramid 1 was artificially depolarized by current injection. Note the opposite direction of potential deviations in pyramids synchronous with field seizure events. In pyramid 1, hyperpolarizing potential deviations indicate their GABAergic origin (the pipette solution contained 20 mM Cl$^-$ corresponding to the Cl$^-$ reversal potential of about -49 mV; note that this value is close to E_{GABA}, see accompanying paper).

decrease in PSP size required for activation of pyramidal cells and *2*) a partial relief of NMDA receptor (NMDAR) channels from Mg^{2+} block. To test this hypothesis, we noninvasively measured E_m (using NMDA channel recordings) in the same

L2/3 pyramidal neurons in control, after appearance of IIS and after observation of the first IS (Fig. 6*A*). Seizures were monitored by field recordings to avoid instabilities of the membrane potential during NMDAR channel measurements.
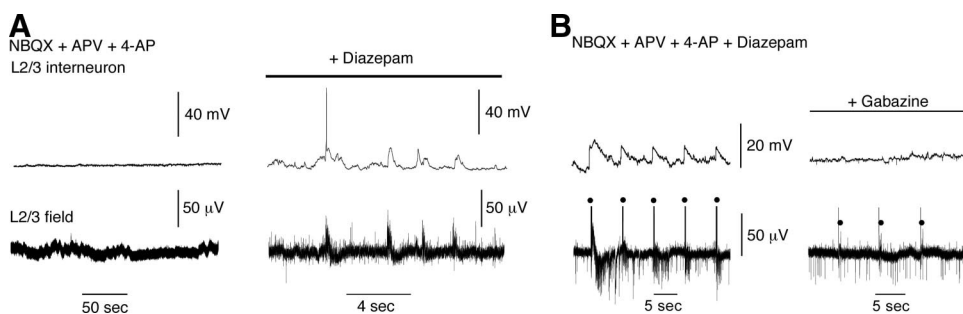
A
NBQX + APV + 4-AP
L2/3 interneuron

+ Diazepam

40 mV   40 mV

L2/3 field

50 µV   50 µV

50 sec   4 sec

B NBQX + APV + 4-AP + Diazepam

+ Gabazine

20 mV

50 µV

5 sec   5 sec

FIG. 4. GABAergic transmission is able to initiate seizures at L2/3. *A*: blockade of the glutamatergic transmission by 4 µM NBQX +40 µM APV before application of 4-aminopyridine (4-AP) prevented seizure initiation (*left*). However, enhancement of efficacy of the GABAergic transmission by 5 µM diazepam resulted in the initiation of spontaneous seizures (*right*). *B*: under similar conditions as in *A*, single stimulations (0.2 ms) via the extracellular electrode positioned in L1 induced strong oscillations (*left*), which were prevented by 10 µM gabazine (*right*).
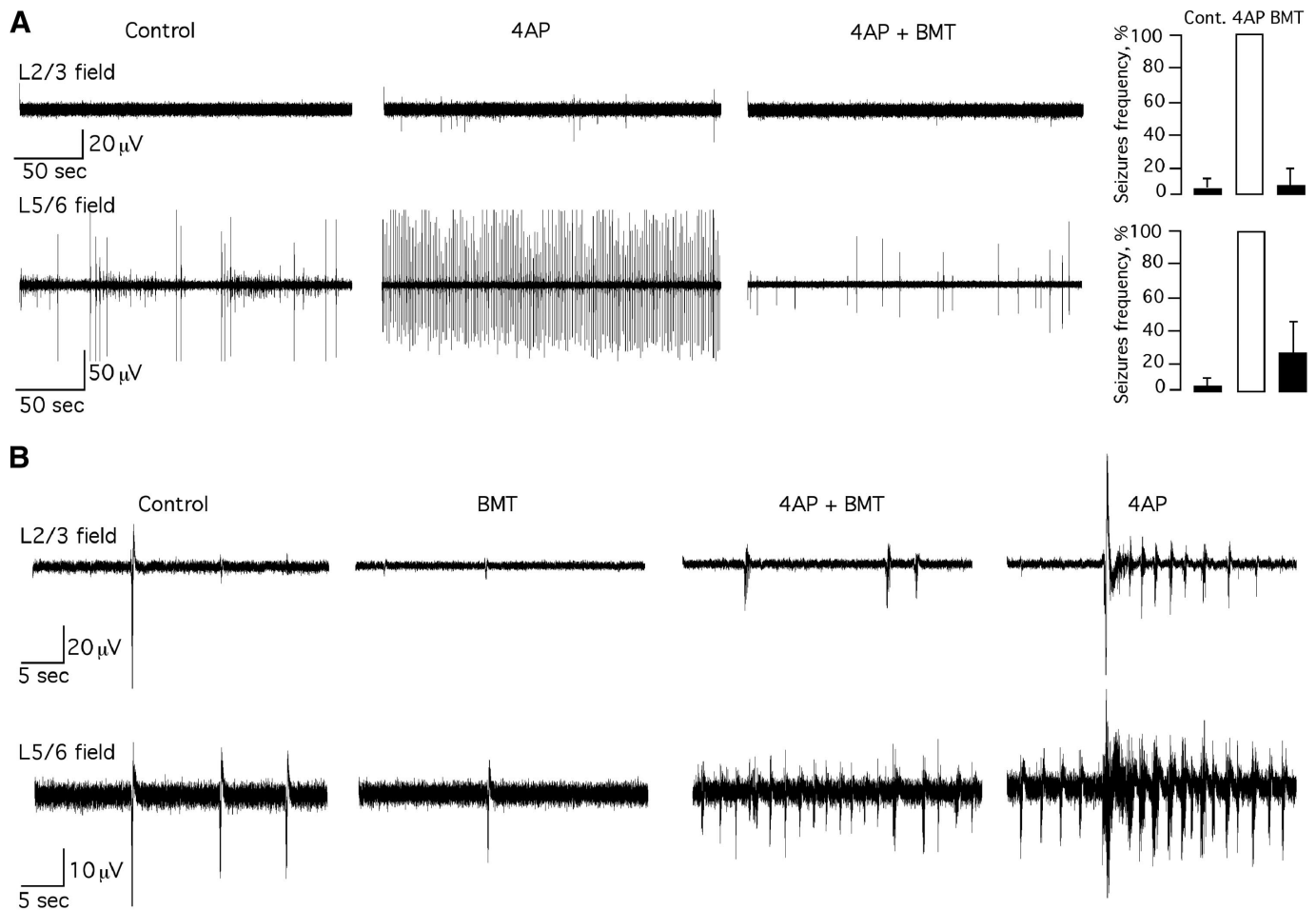
FIG. 5. Seizure initiation and development are related to high $[Cl^-]_i$. *A*: simultaneous field recordings in L2/3 and L5/6. Bumetanide (10 μM) dramatically reduced seizure activity in L5/6 and almost blocked them in L2/3. *B*: when bumetanide was applied before 4-AP, it prevented seizure development from the interictal to ictal phase, ictal events occurring only after bumetanide wash out.

In all eight neurons tested, E_m was significantly depolarized during the initial phase of seizure development (-85.6 ± 4.1 mV in control, and -78.1 ± 3 mV after seizure induction, $P <$ 0.01; paired *t*-test) and depolarized further at the ictal phase (-69.9 ± 3 mV, $P < 0.01$). Similarly, we observed that E_m of L5/6 pyramidal cells was significantly depolarized at the IS phase (-77.7 ± 2.4 mV in control and -66.8 ± 2.05 mV at the ictal phase, $n = 9$, $P < 0.03$; paired *t*-test).

To delineate the contribution of synaptic activity into the gradual membrane depolarization, we blocked synaptic transmission by a mixture of antagonists (APV + NBQX + picrotoxin) after initiation of IISs. Figure 6C shows that pyramidal cells were depolarized at the initial phase of seizure development (-82.3 ± 0.8 mV in control, and -74 ± 2 mV during seizures, $n = 5$, $P < 0.01$, paired *t*-test). However, this depolarization was completely abolished by application of antagonists (-86.5 ± 1 mV). These results verify also that the continuous presence of 4-AP per se does not promote the membrane depolarization.

Bumetanide reduced seizure activity and abolished the persistent neuronal depolarization (-68.56 ± 1.55 mV at ictal phase and -81.38 ± 3.40 mV after bumetanide, $n = 6$; $P <$ 0.03, paired *t*-test). Interestingly, E_{GABA} measured with single GABA channel recordings was not modified during seizure development ($E_{GABA} = -51.20 \pm 3.7$ mV in control and

-53.82 ± 5.72 mV during seizures, $n = 7$; $P = 0.72$, paired *t*-test, data not shown).

To verify that the network depolarization is independent of the type of convulsive agent, we applied carbachol (50 μM), an agonist of cholinergic receptors, and DPCPX (200 nM), an A1 adenosine receptor antagonist. Although less efficient than 4-AP, this mixture generated a pronounced increase in network activity (see *inset* to Fig. 6E, *left*; 60 min after drug application) and a significant depolarization of pyramidal cells (-84 ± 1.4 mV in control and $-72.7 \pm$ 1.4 mV after seizure induction; $n = 5$; $P < 0.01$). Moreover, after blockade of synaptic transmission in the same experiments by NBQX + APV + PTX, E_m recovered its control value (-81.8 ± 0.9 mV).

It was unclear, however, whether the membrane depolarization of 10–15 mV may result in a significant change in the pyramidal cell excitability. To address this issue, we performed whole cell current-clamp recordings from a L2/3 pyramidal cell held at -80 mV while stimulating nerve fibers in L1 (Fig. 6F). Single shocks (0.2 ms) induced polysynaptic subthreshold PSPs in the pyramidal cell (Fig. 6F, *top*). However, when the pyramidal cell was depolarized by ~10 mV, similar extracellular stimulation evoked reliable firing of the pyramidal cell (Fig. 6F, *bottom*).
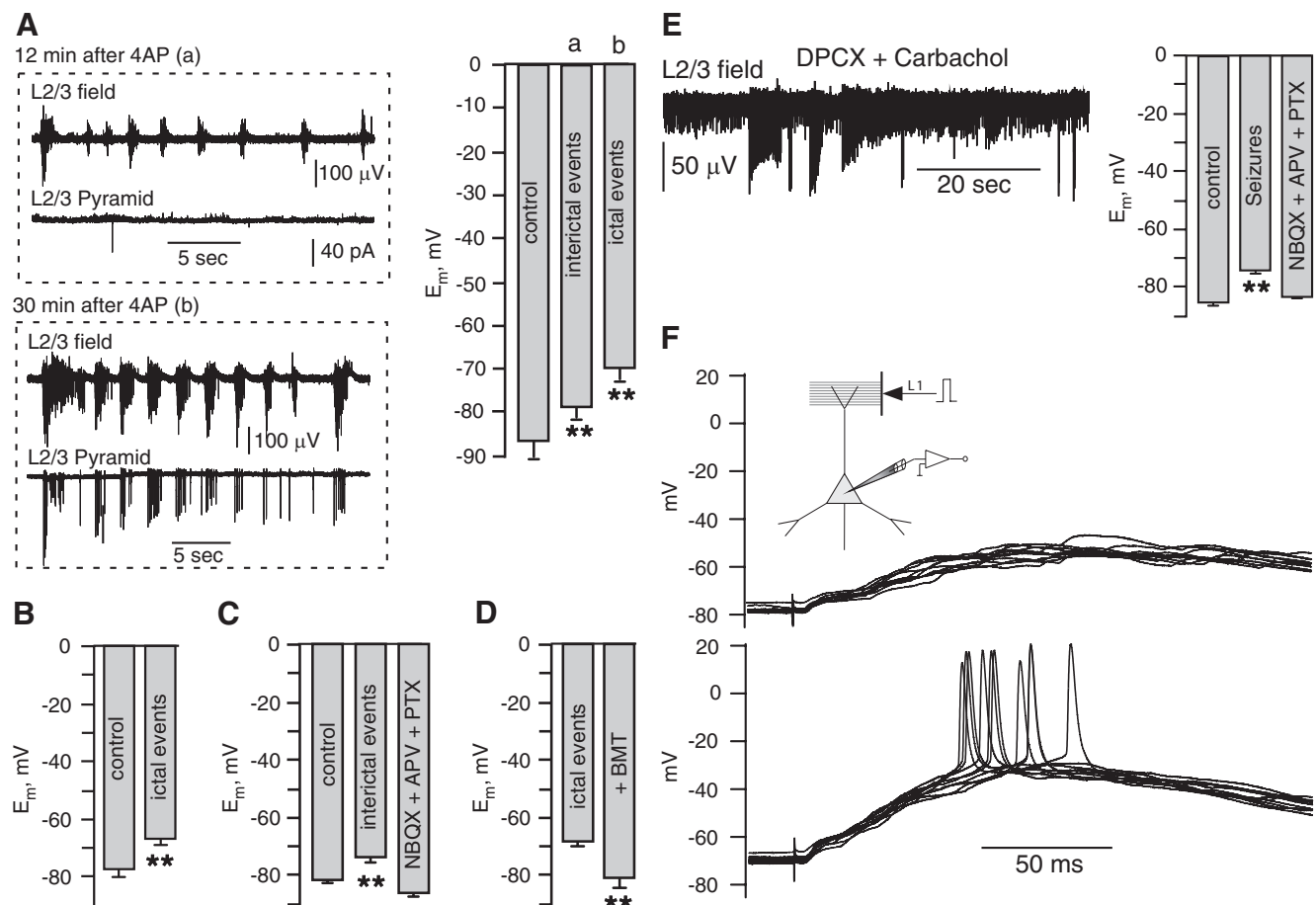
FIG. 6. Seizure development is associated with the network depolarization. *A*: L2/3 pyramidals were quiescent at interictal phase (*inset a*). With the recurrence of seizures, the ictal phase developed, maintained by activity of the whole neuronal network, including L2/3 pyramidal 1 (*inset b*). This transition was related to the progressive depolarization of neuronal network: E_m of L2/3 pyramidal cells depolarized during development of seizures. E_m was significantly depolarized during interictal seizures and depolarized further at the ictal-like seizure phase. *B*: L5/6 pyramidal cells were also depolarized during development of seizures. *C*: depolarization induced by seizures was completely abolished by application of a mixture of antagonists [40 μM APV + 4 μM NBQX + 75 μM picrotoxin (PTX)] or (*D*) by10 μM of bumetanide. *E*: membrane depolarization was not a consequence of a seizure initiation model. The network activity was induced by a mixture of carbachol (50 μM) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (200 nM; *left*). Pyramidal cells were significantly depolarized and E_m recovered to its negative value after application of NBQX + APV + PTX (*right*). *F*: membrane depolarization of 10–15 mV resulted in a significant change in pyramidal cell excitability. A pyramidal cell was held at −80 or −70 mV while stimulating nerve fibers in L1. At −80 mV, single shocks (0.2 ms) induced polysynaptic subthreshold excitatory postsynaptic potentials (EPSPs) in the pyramidal cell (*top*). At –70 mV, similar extracellular stimulation evoked reliable firing of the cell (*bottom*).

Thus the intense synaptic activity underlying seizure initiation results in the gradual membrane depolarization. Such depolarization enhances excitability of pyramidal cells and therefore may play a principal role in the process of seizure development.

DISCUSSION

Our study provides the first systematic layer and neuron type–specific analysis of cellular mechanisms of seizure initiation and development in the neonatal neocortex. We show that, in L2/3, GABAergic interneuron network plays a major role in the process of seizure initiation. GABAergic transmission is excitatory in the range of potentials wide enough to provide activation of interneurons in all layers and pyramidal cells in L5/6 but not in L2/3. However, the intense synaptic activity during interictal events results in the gradual network depolarization, which underlies the involvement of pyramidal cells in network oscillations and consequent amplification of seizures.

Interneuron network triggers interictal seizures in neonatal neocortex

The contribution of GABAergic transmission to seizure generation has been intensively studied on neonatal rodent hippocampal formation (Dzhala and Staley 2003; Dzhala et al. 2005, 2008; Khalilov et al. 1999, 2003, 2005; Khazipov and Holmes 2003; Khazipov et al. 2004). During the first postnatal week, a high $[Cl^-]_{in}$ defines its excitatory role (Tyzio et al. 2007, 2008; for review, Ben-Ari 2002; Ben-Ari et al. 2007). Obviously, having profoundly depolarizing GABAergic transmission, the neonatal neuronal network is to a large extent more vulnerable to seizures than the mature one. Although GABA depolarizes immature neurons in neocortex (Owens et al. 1996; Yamada et al. 2004; see accompanying paper), there is no evidence that this action directly impacts seizures.

Our results show that GABAergic interneuron network plays a profound role in the process of seizure initiation. The initiating phase of seizures is characterized by IISs generated in L5/6 and supported by activation of both L5/6 interneurons and pyramidal cells. IISs propagate to L2/3 via activation of L2/3 interneurons but not pyramidal cells, which are mostly quiescent at this phase. In L2/3, the interneuron network (at least some fraction of it) is much more excitable normally than the pyramidal one. For instance, APs can be readily initiated in the intact L2/3 interneurons by a pure GABAergic transmission that is not the case for L2/3 pyramidal cells (Rheims et al. 2008). The likely reason for this is that, although E_m is quite similar in L2/3 interneurons and pyramidal cells, the latter have a much more positive AP activation threshold (Rheims et al. 2008). Therefore at an early stage, L2/3 IIS consist mostly of synchronous firing of interneurons. This firing is reflected in pyramidal cells as strong depolarizations provided by GABAergic transmission (see Fig. 3).

Clearly, glutamatergic transmission is important for the interneuron excitation since IISs have not been induced by 4-AP under blockade of glutamate-activated receptors. However, being induced, IISs are not as sensitive to glutamate receptors antagonists. This suggests that glutamatergic transmission provides interneurons the fraction of initial excitation necessary to trigger seizures. Because of their lower AP activation threshold (Rheims et al. 2008), L5/6 pyramidal cells are more excitable than L2/3 ones and are involved in the process of seizure initiation. Therefore they provide the excitatory input required to activate the interneuron network. In contrast, it is unlikely that L2/3 pyramidal cells contribute significantly to this early drive since they are mostly quiescent.

The central role of the interneuron network suggests that shifting the action of GABAergic transmission from excitatory to inhibitory may block seizure development. It has been shown that the NKCC1 blocker bumetanide, which decreases $[Cl^-]_{in}$ in immature neurons, blocks hippocampal seizures (Dzhala et al. 2005, 2008) and may thus act in synergy with $GABA_ARs$ modulators that are recommended as the first line treatment of neonatal seizures (Bartha et al. 2007; Dzhala et al. 2008; Wheless et al. 2005, 2007). As observed in hippocampus, bumetanide dramatically reduces neocortical seizure activity and prevents development from the interictal to ictal phase (see Fig. 5).

Neuronal depolarization underlies transition of network oscillations to ictal seizures

Seizure development is manifested by transition from interictal to ictal seizure events. This transition is accompanied by involvement of L2/3 pyramidal cells into network oscillations (Figs. 2A and 6A). We show that one reason for the activation of L2/3 pyramidal cell firing is depolarization of the whole neuronal network. The amount of this depolarization correlates with the time-dependent intensification of seizure events, depends on the level of synaptic activity, and is abolished by blockade of synaptic transmission. Importantly, the network depolarization that is close to steady state persists between seizure events and differs from the transient depolarization induced by enhancement of the extracellular K^+ concentration during seizures (Avoli et al. 1996; Hablitz and Heinemann 1987; Lucke et al. 1995; Stringer 1998). Indeed, the clearance of extracellular

K^+ is only a few seconds longer in the immature brain than in the older one despite an altered regulation of extracellular K^+ (Connors and Ransom 1984; Stringer 1998). Furthermore, the neuronal depolarization was not related to the direct action of 4-AP on E_m since it was either completely abolished by blockade of synaptic transmission or observed with another experimental model (Fig. 6, C and E). The mechanism of network depolarization is yet unknown. It is not associated with the intracellular accumulation of Cl^- ions. It is possible, however, that the intensive synaptic activity during seizures results in an increase in ambient GABA concentration and a consequent membrane depolarization.

We conclude that the increasing involvement of glutamatergic transmission into network oscillations, powered by neuronal depolarization, provides a positive feedback highly amplifying seizure generation.

Functional implications

Our results show that, in the neonatal neocortex, seizures are initiated in deep layers and thereafter propagate to L2/3 where they are triggered by synchronization of GABAergic interneurons. Although the precise mechanism of such synchronization remains unclear, we suggest that it may be promoted by spontaneous episodes of hyperactivity (sensory, motor, etc.) at A functionally normal neocortical network. Moreover, whereas the link between interictal events and epileptogenesis remains a matter of debate (Avoli et al. 2006; de Curtis and Avanzini 2001; Staley and Dudek 2006), our data suggest that IISS and neuronal network depolarization may play a pivotal role in subsequent seizure development and epileptogenesis. Although these conclusions are made for a limited developmental stage (P6–P9), we suggest that the interneuron network may represent a key target for pharmacological interventions aiming to prevent initiation and development of neocortical seizures. However, while developing and administrating such medications, rigorous precautions should be made, given that drugs affecting GABAergic transmission may be beneficial for the neocortical network but not necessarily for the hippocampal one (see Supplemental Fig. 2).

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