Brain Advance Access published March 23, 2011

BRAIN
A JOURNAL OF NEUROLOGY

Neuronal chloride accumulation and excitatory GABA underlie aggravation of neonatal epileptiform activities by phenobarbital

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Phenobarbital produces its anti-epileptic actions by increasing the inhibitory drive of γ-aminobutyric acid. However, following recurrent seizures, γ-aminobutyric acid excites neurons because of a persistent increase of chloride raising the important issue of whether phenobarbital could aggravate persistent seizures. Here we compared the actions of phenobarbital on initial and established ictal-like events in an *in vitro* model of mirror focus. Using the *in vitro* three-compartment chamber preparation with the two hippocampi and their commissural fibres placed in three different chambers, kainate was applied to one hippocampus and phenobarbital contralaterally, either after one ictal-like event or after many recurrent ictal-like events that produce an epileptogenic mirror focus. Field, perforated patch and single-channel recordings were used to determine the effects of γ-aminobutyric acid and their modulation by phenobarbital, and alterations of the chloride cotransporters were investigated using sodium–potassium–chloride cotransporter 1 and potassium chloride cotransporter 2 antagonists, potassium chloride cotransporter 2 immunocytochemistry and sodium–potassium–chloride cotransporter 1 knockouts. Phenobarbital reduced initial ictal-like events and prevented the formation of a mirror focus when applied from the start. In contrast, phenobarbital aggravated epileptiform activities when applied after many ictal-like events by enhancing the excitatory actions of γ-aminobutyric acid due to increased chloride. The accumulation of chloride and the excitatory actions of γ-aminobutyric acid in mirror foci neurons are mediated by the sodium–potassium–chloride cotransporter 1 chloride importer and by downregulation and internalization of the chloride-exporter potassium-chloride cotransporter 2. Finally, concomitant applications of the sodium–potassium–chloride cotransporter 1 antagonist bumetanide and phenobarbital decreased excitatory actions of γ-aminobutyric acid and prevented its paradoxical actions on mirror focus. Therefore, the history of seizures prior to phenobarbital applications determines its effects and rapid treatment of severe potentially epileptogenic-neonatal seizures is recommended to prevent secondary epileptogenesis associated with potassium chloride cotransporter 2 downregulation and acquisition of the excitatory γ-aminobutyric acid phenotype.

Received July 23, 2010. Revised January 21, 2011. Accepted January 21, 2011.

Keywords: anti-epileptic drug; GABA; paediatric epilepsy; ion channel electrophysiology; neuropharmacology

Abbreviations: APV = d-2-amino-5-phosphopentanoate; CNQX = 6-cyano-7-nitroquinoxaline-2,3 dione; DIOA = R-(+)-[(2-*n*-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid; GABA = γ-aminobutyric acid; KCC2 = potassium chloride cotransporter 2; NKCC1 = sodium–potassium–chloride cotransporter 1

Introduction

The γ-aminobutyric acid (GABA) acting anti-epileptic drug phenobarbital is the drug of first choice to treat neonatal seizures (Wheless *et al.*, 2007; Bassan *et al.*, 2008). However, phenobarbital is less efficient for severe seizures (Painter *et al.*, 1999), aggravates EEG discharges (Boylan *et al.*, 2002; Guillet and Kwon, 2007) and is associated with high rates of complications (Yanay *et al.*, 2004; Kaindl *et al.*, 2008). It is, therefore, important to determine why phenobarbital effects are altered by recurrent seizures and whether these changes are due to changes in the action of GABA. Indeed, neurons respond to recurrent seizures with an increase in chloride, and by a shift in the action of GABA from inhibitory to excitatory (Cohen *et al.*, 2002; Dzhala *et al.*, 2005; Khalilov *et al.*, 2005; Huberfeld *et al.*, 2006; Kahle *et al.*, 2008; Nardou *et al.*, 2009). Two mechanisms have been suggested to underlie these changes: enhanced activity of the chloride importer sodium–potassium–chloride cotransporter 1 (NKCC1) (Dzhala *et al.*, 2005, 2008; Brandt *et al.*, 2010) and a down-regulation of the chloride exporter potassium-chloride cotransporter 2 (KCC2) (Rivera *et al.*, 2004; Jin *et al.*, 2005; Pathak *et al.*, 2007).

To investigate the effects of seizures on GABA and phenobarbital action, we used an *in vitro* chamber preparation (Khalilov *et al.*, 1997, 2003) composed of two intact neonatal hippocampi and their commissural connections placed in three independent compartments. Applications of a convulsive agent to one hippocampus (kainate) generate ictal-like events that propagate to the contralateral hippocampus, transforming it into a chronic epileptic mirror focus (Khalilov *et al.*, 2003, 2005). This preparation, therefore, enables application of a convulsive agent to one hippocampus and an anti-epileptic drug to the other either from the start or after repeated propagations of ictal-like events from the contralateral hippocampus. We report that phenobarbital reduces initial ictal-like events but aggravates them when applied after the formation of a mirror focus. We also show that GABA excites epileptic neurons due to high intracellular chloride mediated by a combined action of the NKCC1 chloride importer but also a down-regulation of KCC2, leading to strong excitatory actions of GABA that phenobarbital exacerbates. In keeping with Dzhala *et al.* (2008), we also show that the diuretic inhibitor of NKCC1 bumetanide ameliorates the situation and combined applications of the diuretic and phenobarbital are efficient in reducing seizure severity in particular, when the diuretic is applied before phenobarbital (at an early stage). Therefore, the anti-epileptic actions of phenobarbital depend on seizure history prior to treatment and rapid treatment with a diuretic may be a useful therapeutic tool to prevent excessive chloride accumulation and increase the efficiency of the anti-epileptic actions of phenobarbital.

Materials and methods

Further details of the materials and methods used can be found in the online Supplementary material.

Animals

All experiments were carried out in accordance with the European Communities Council Directive of the 24 November 1986 (86/609/EEC).

Tissue preparation

Experiments were performed on neonatal Wistar rats (postnatal days P7–P8), C57BL/6 wild-type and NKCC1$^{-/-}$ mice (P6–P7). The intact and slice hippocampal preparations, and the three independent compartments chamber and experimental conditions have been described previously (Khalilov *et al*., 2003).

Electrophysiology

The detailed electrophysiological recordings, data analysis and pharmacological agents used in this study are described in the Supplementary Material. In brief, extracellular field potentials and multi-unit activities were recorded in the hippocampal slices and in the intact hippocampal preparations *in vitro* using tungsten wire electrodes. Electrical stimulations were performed with a bipolar electrode. GABA was focally applied by a picospritzer from a glass pipette. Patch-clamp recordings in different configurations were collected using an Axopatch 200B and MultiClamp 700B amplifiers (Axon Instruments, USA).

Immunohistochemistry of KCC2

Immunohistochemistry is described in detail in the Supplementary Material. KCC2 antibody is highly specific as attested by the lack of labelling in KCC2$^{-/-}$ (Supplementary Fig. 2).

NKCC1$^{-/-}$ genotyping

The detailed genotyping is described in the Supplementary material. In brief, C57BL/6 knockout mice for NKCC1 (provided by Pr C Hubner, Germany) were evaluated by polymerase chain reaction using standard protocols. The littermate wild-type mice were used as controls.

Results

Phenobarbital reduces the severity of initial ictal-like event but aggravates ictal-like events generated by an epileptogenic mirror focus

Using the triple chamber, we applied kainate (400 nM for 3 min, every 20 min) to one hippocampus (referred to as the ipsilateral

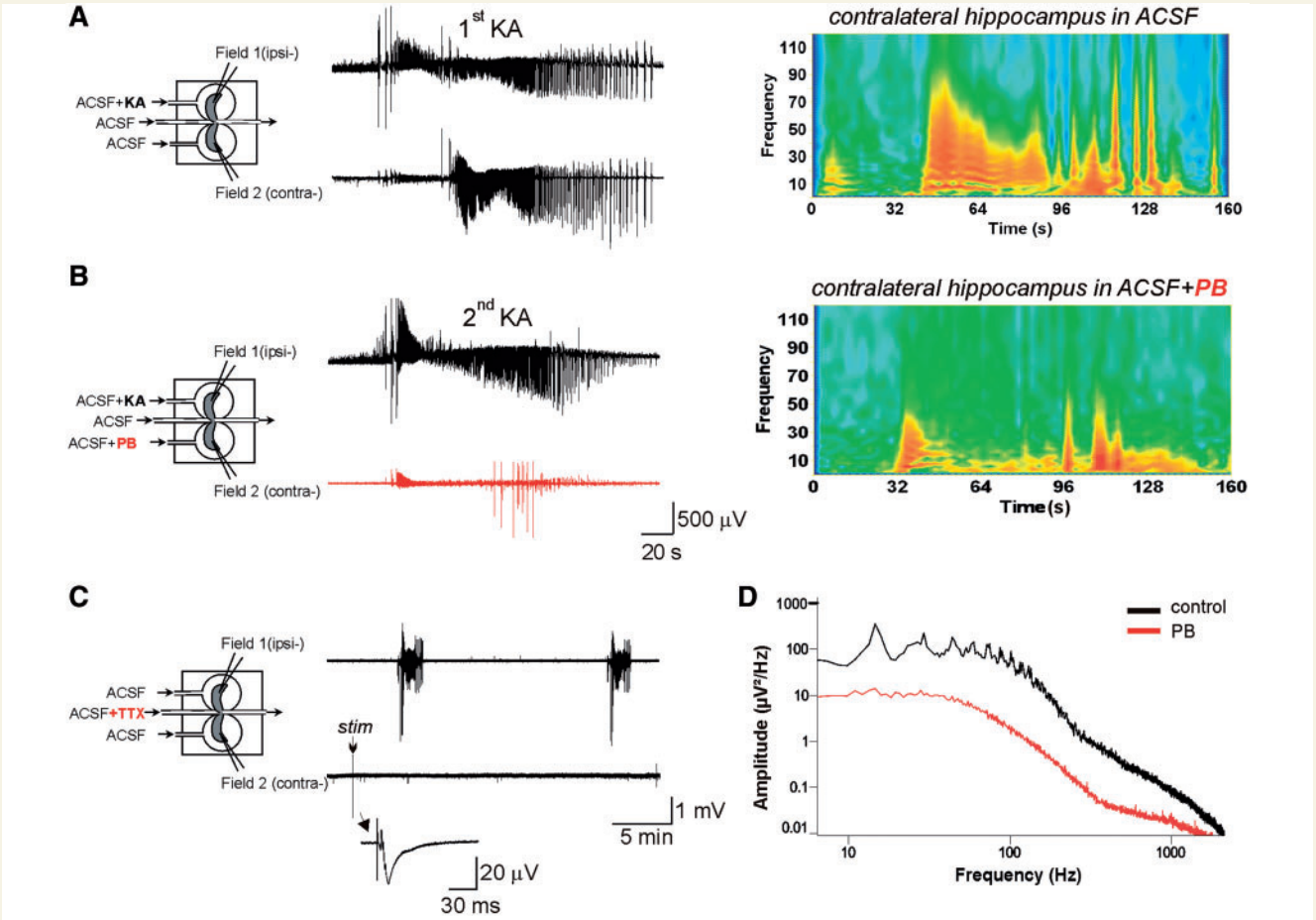

Figure 1 Phenobarbital prevents the formation by ictal-like events of an epileptogenic mirror focus. (**A–C**) The scheme depicts the triple chamber preparation with the two intact hippocampi and their connecting inter-hemispheric commissure in independent chambers (left). Field recordings were made in the two hippocampi. (**A**) Kainate (KA) applied to one hippocampus (ipsilateral = ipsi-, grey) generated seizure activity referred to as ictal-like events that propagated to the contralateral naïve hippocampus (contra-). The ictal-like events included γ-oscillations (>40 Hz) as shown in the time frequency analysis in the right side of the figure. (**B**) Application of phenobarbital (PB) to contralateral hippocampus (100 µM, during 15 min) before second application of kainate decreased the ictal-like event and almost completely blocked γ-oscillations. (**C**) After 15 transient applications of kainate on the ipsilateral side with continuous application of phenobarbital on contralateral side, disconnection of the two hippocampi by applications of tetrodotoxin to the commissural chamber revealed that the contralateral side generated neither spontaneous nor evoked (by electrical stimulation = stim) ictal-like events. (**D**) Power spectra showing decreased amplitude of the population activity of the contralateral side treated with phenobarbital (PB) during application of kainate on the ipsilateral side. ACSF = artificial cerebrospinal fluid.

side), and recorded the activity from both the kainate treated and the other hippocampus naïve for kainate (referred to as the contralateral side). In keeping with our earlier observations (Khalilov *et al.*, 2003, 2005), kainate generated an ictal-like event with large-amplitude (1–2 mV) discharges (Fig. 1A, see also Khalilov *et al.*, 2003). These ictal-like events propagated to the contralateral hippocampus where they generated similar activities. In keeping with our earlier observations (Khalilov *et al.*, 2003, 2005), the duration of each ictal-like event increased after repeated applications of kainate (from 82.2 ± 9.6 to 102.7 ± 7.8 s, $P < 0.01$, $n = 23$). In further agreement with these earlier studies, ictal-like events included γ-oscillations (40–120 Hz) that were also progressively increased with recurrent applications of kainate to the ipsilateral hippocampus (Khalilov *et al.*, 2005). After ~15 applications, the contralateral hippocampus generated spontaneous ictal-like events ($n = 23$) when disconnected from the treated hippocampus (Khalilov *et al.*, 2003, 2005). We refer to this as a newly formed epileptogenic mirror focus. Using this paradigm, we compared two experimental situations:

(i) When applied from the beginning on the initial ictal-like events, phenobarbital prevents the formation of a mirror focus. Application of phenobarbital (100 µM) to the contralateral hippocampus reduced the total power of ictal-like events and the occurrence of γ-oscillations in every experiment (Fig. 1B and D, $n = 6$). γ-Oscillations shifted from >60 Hz recorded prior to phenobarbital application to <20 Hz in presence of phenobarbital. This effect is a good indicator of phenobarbital action as γ-oscillations constitutes a signature of severe seizures and their sites of origin (Bragin *et al.*, 1999; Khalilov *et al.*, 2005; Jacobs *et al.*, 2008*a*). When the two hippocampi were disconnected after

15 unilateral kainate applications, the ipsilateral side generated spontaneous ictal-like events but the contralateral hippocampus treated with phenobarbital did not, i.e. a mirror focus was not formed (Fig. 1C, $n = 8$). Therefore, when applied early, phenobarbital reduces ictal-like events severity and prevents the formation of a mirror focus.

(ii) Phenobarbital aggravated ongoing ictal-like events generated by the mirror focus. When phenobarbital (100 μM) was first applied after 14 applications of kainate to the ipsilateral hippocampus, phenobarbital increased the evoked (Fig. 2A–C, $n = 4$) and spontaneous (Fig. 2D, $n = 4$) ictal-like events generated by the isolated contralateral hippocampus once disconnected from the kainate treated side. The average power of spontaneous ictal-like events in isolated mirror focus hippocampi after phenobarbital treatment increased by 24.2 ± 7.3% ($P < 0.01$, $n = 4$, Fig. 2E and F).

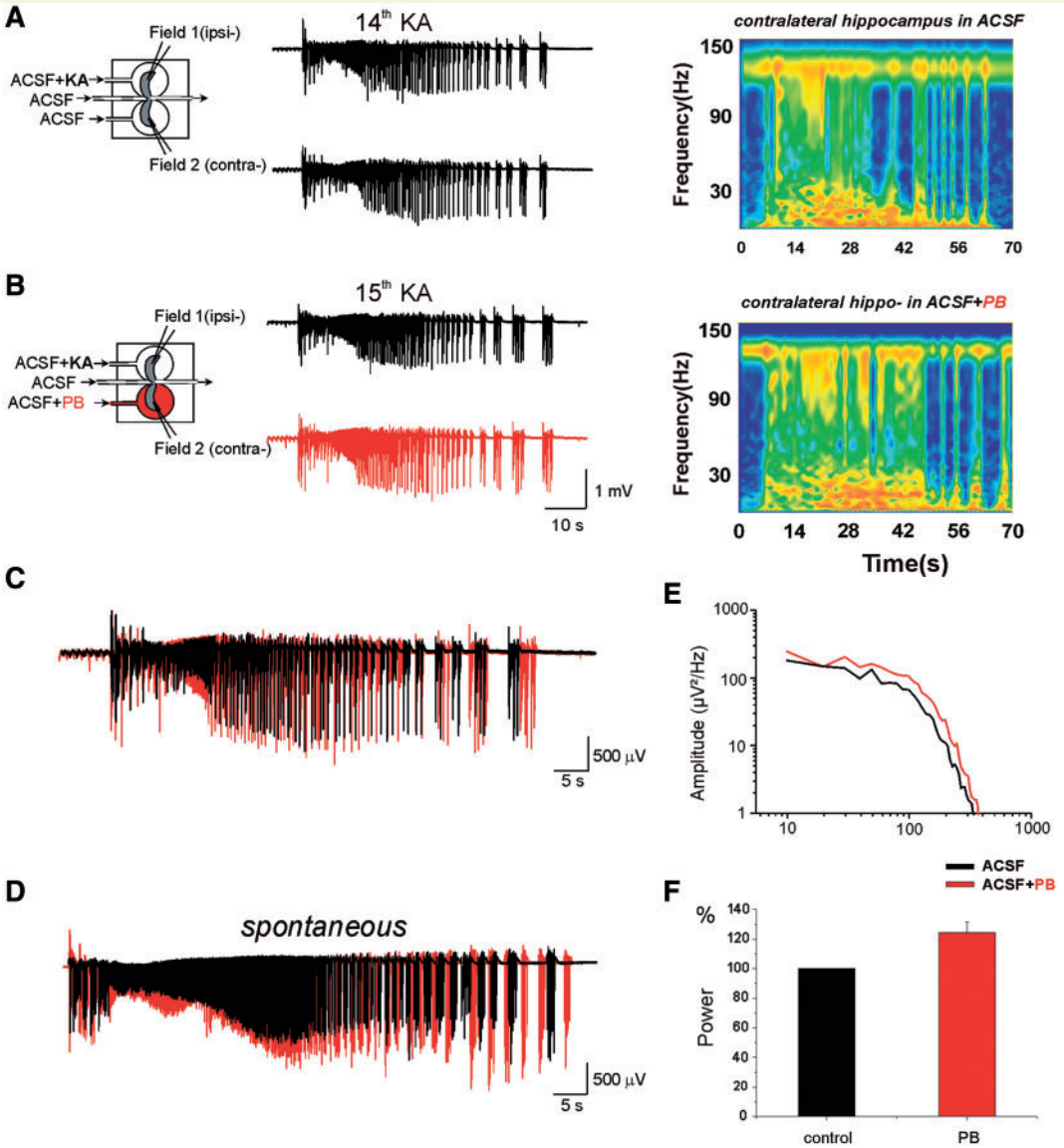

Figure 2 Late application of phenobarbital fails to block the ictal-like event and γ-oscillations. Kainate (KA) was applied repeatedly (every 20 min) ×15 to one hippocampus (ipsilateral = ipsi-) and artificial cerebrospinal fluid (ACSF) to the contralateral naïve hippocampus (contra-). (**A**) Fourteenth ictal-like event is generated on the ipsilateral side (grey) and propagated to the contralateral side (black). The time frequency analysis (on the right, hippocampus = hippo-) shows γ-oscillations (in the >90 Hz band). (**B**) phenobarbital (PB) applied to the contralateral side 15 min before the 15th application of kainate to the ipsilateral side failed to block propagating ictal-like events (red). The time frequency also shows γ-oscillations. (**C**) Superimposed ictal-like events (from **A** and **B**) recorded from the contralateral side before (black) and after phenobarbital application (red). Phenobarbital produced a small increase of the amplitude and duration of ictal-like events. (**D**) Superimposed ictal-like events generated spontaneously in the contralateral side after formation of mirror focus before (black) and after (red) phenobarbital application. Phenobarbital also increased the amplitude and duration of spontaneous ictal-like events. (**E**) Power spectra of spontaneous ictal-like events before and after phenobarbital treatment (from **D**). (**F**) Average power histogram of spontaneous ictal-like events before and after phenobarbital. Phenobarbital application significantly increased power of ictal-like events (by 24.2 ± 7.3%, $n = 4$, $P < 0.01$).

Phenobarbital reduces ongoing neuronal activity and giant depolarizing potentials

In control conditions, the physiological pattern of the neonatal hippocampal network activity is characterized by spontaneous network-driven giant depolarizing potentials (Ben-Ari *et al.*, 1989) that occur spontaneously and can be evoked by electrical stimulation. Spontaneous action potentials (spikes) and bursts of network-driven, high frequency action potentials representing giant depolarizing potentials from multiple cells were recorded (Fig. 3A). Bath application of phenobarbital (100 μM) did not affect the occurrence of giant depolarizing potentials but substantially reduced their amplitude. A dose–response curve revealed a substantial (~25%) reduction at 100 μM—the dose used subsequently—and a full blockade with higher concentrations (Fig. 3D and E). To determine the effects of phenobarbital on the network activity, we measured spontaneous firing rates of CA3 hippocampal neurons using metal electrodes that detect spikes generated by adjacent neurons. Multi-unit activity was measured from the same populations of neurons before and during application of phenobarbital (Fig. 3B). Bath application of phenobarbital dramatically decreased spontaneous neuronal firing rate by 42% (to 58.4 ± 11.5%) (Fig. 3A–C, $P < 0.001$, $n = 5$). In whole cell recordings from CA3a pyramidal cells focal GABA applications to CA3c region generated giant depolarizing potentials. The effects of phenobarbital were dose dependent with no effect at 1 μM and a full block at 1 mM (Fig. 3D and E, $n = 5$). Similar focal application of GABA generated 2–3 action potentials in cell attached recordings (Fig. 3F). Bath applications of phenobarbital (100 μM) reduced the number of action potentials (from 2.7 ± 0.2 to 1.9 ± 0.2, $n = 7$ neurons, 10 stimuli per neuron, $P < 0.05$, Fig. 3F). Therefore, phenobarbital reduces ongoing neuronal activity and GABA excitatory actions.

Phenobarbital aggravates interictal-like events generated by mirror focus neurons and augments excitatory actions of GABA

Slices prepared from the isolated mirror focus hippocampi generate spontaneous interictal-like events in the 0.1–0.25 Hz range (Khalilov *et al.*, 2003, 2005; Nardou *et al.*, 2009). Phenobarbital

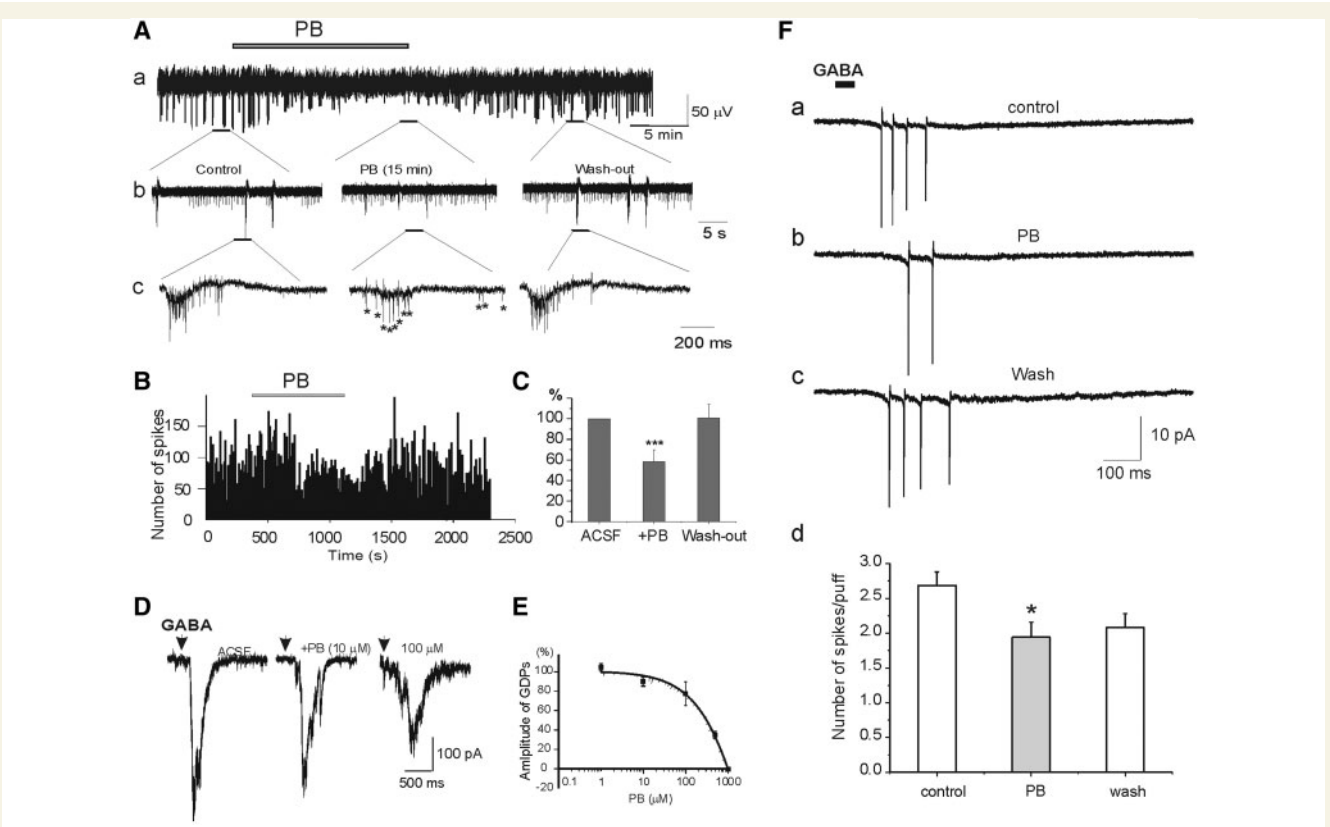

Figure 3 Phenobarbital reduces ongoing activity and excitatory actions of GABA in naïve neurons. (**Aa**) Extracellular recording of multi-unit activity showing the reduction of giant depolarizing potentials and spikes by phenobarbital. Ten hertz low pass filter. Non-filtrated faster display traces are depicted below (**Ab** and **Ac**). (**B**) Quantification of recorded spikes (*example of quantified spikes) from **A**, Bin = 20 s. (**C**) Normalized multi-unit activity frequency ($n = 5$, $***P < 0.001$). (**D**) Phenobarbital dose-dependently reduced the amplitude of giant depolarizing potentials generated by focal application of GABA. Normalized dose–response curve and quantification in **E**. (**Fa–c**) Cell attached recordings of spikes evoked by focal application of GABA as in **D.** Note the reduction by phenobarbital the number of spikes evoked by GABA. Quantification in **Fd**, $*P < 0.05$. ACSF = artificial cerebrospinal fluid; PB = phenobarbital.

augmented the number of interictal-like events (Fig. 4B by $71 \pm 10\%$, $n = 20$, $P < 0.001$) and the number of spikes generated during and between interictal-like events (Fig. 4C, $65 \pm 11\%$, $n = 20$, $P < 0.001$). Therefore, phenobarbital aggravates epileptiform activities in the mirror focus.

Are the pro-epileptic actions of phenobarbital mediated by the enhanced GABAergic excitation? We compared the effects of phenobarbital (100 μM) on control and mirror focus neurons using cell attached recordings. Focal GABA applications generated more action potentials in mirror focus neurons than in control ones (4.0 ± 0.3, $n = 6$ and 2.7 ± 0.2, $n = 7$, respectively, $P < 0.001$; Fig. 5A*a–d* to compare with Fig. 3F*a–d*), suggesting a strengthening of GABA excitation in mirror focus neurons. In contrast to control neurons, bath applications of phenobarbital increased the number of action potentials in mirror focus ones (Fig. 5A*a–d* from 4.0 ± 0.3 to 5.4 ± 0.4, $n = 6$, $P < 0.01$). Phenobarbital also increased significantly the perforated patch-clamp recorded post-synaptic currents generated by focal applications of GABA (Fig. 5B*a–c*, $n = 4$): area from 177.5 ± 9.1 to 259.2 ± 7.4 nA/s ($P < 0.001$); amplitude: from 133.4 ± 3.8 to 162.9 ± 3.4 pA ($P < 0.001$); decay time: from 1953.7 ± 119.8 to 2359 ± 83.9 ms ($P < 0.001$) half-width: from 1346.7 ± 43.2 to

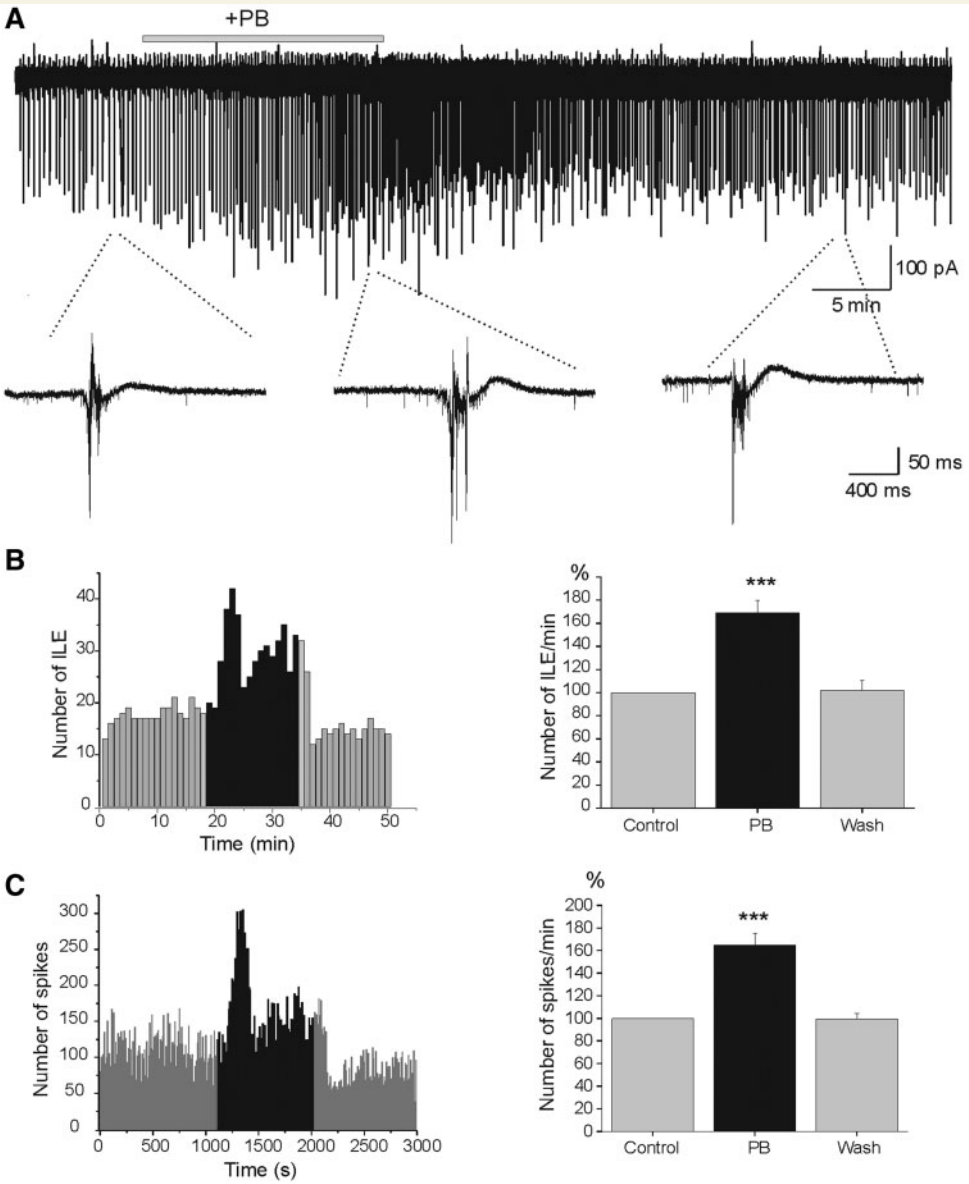

Figure 4 Phenobarbital aggravates interictal-like events (ILE) generated by slices obtained from mirror focus hippocampi. Slices were obtained from intact hippocampus after the formation of a mirror focus. (**A**) Slices generated interictal-like events continuously that phenobarbital (100 μM) aggravated reversibly. (**B**) Quantification of ictal-like events from single experiment (*left*, bin = 1 min). *Right*: the average quantification histogram of ictal-like events. (**C**) Quantification of spikes from single experiment (*left*, bin = 20 s). *Right*: the average quantification histogram. Note that phenobarbital (PB) increased frequency of ictal-like events (by $71 \pm 10\%$, $n = 20$, $^{***}P < 0.001$) and spikes (by $68 \pm 20\%$, $n = 7$, $^{***}P < 0.001$).

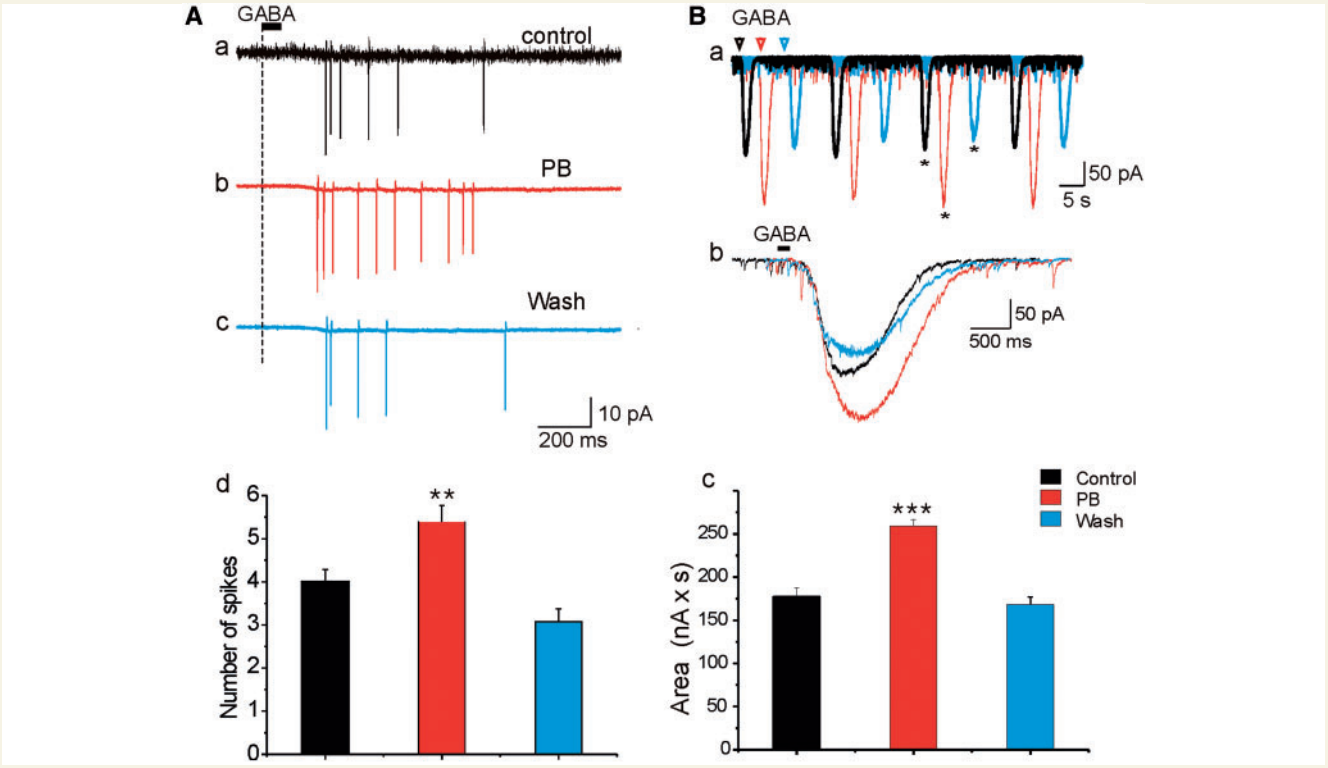

Figure 5 Phenobarbital enhances GABA excitation in mirror focus neurons. (**A**) Cell attached recordings from mirror focus neurons to illustrate the increased number of action potentials generated by focal application of GABA in the presence of phenobarbital (PB) (**Aa–Ac**). Quantified group data are shown in **Ad**, $^{**}P < 0.01$. (**Ba**) Superimposed traces from perforated patch clamp recordings of post-synaptic currents generated by focal application of GABA (every 20 s, arrowheads) in the presence of CNQX and APV before (black), during (red) application of phenobarbital and after wash out (blue). (**Bb**) Traces at higher magnification from **Ba** (asterisks), to illustrate the increased amplitude and duration of post-synaptic currents generated by focal applications of GABA in the presence of phenobarbital. (**Bc**) Histogram representing the quantifications of area ($n = 4$, $^{***}P < 0.001$).

1557.2 ± 14.3 ms ($P < 0.001$). The rise time (10–90%) was not changed significantly (from 523 ± 18.6 to 562.8 ± 10.3 ms, $P = 0.53$). Therefore, phenobarbital augments GABA signals in mirror focus neurons possibly by a chronic increase of intracellular chloride. We next examined the roles of chloride cotransporters NKCC1 and KCC2 in these changes.

NKCC1 is preserved and KCC2 is downregulated in mirror focus neurons

Seizures beget seizures in NKCC1$^{-/-}$

In our earlier study, we showed that continuous applications of the diuretic NKCC1 antagonist bumetanide do not prevent the formation of a mirror focus by ictal-like events (Nardou *et al.,* 2009). Here, we used the NKCC1 knockout mice (NKCC1$^{-/-}$) in which the transporter has been genetically invalidated (Fig. 6A). The two interconnected intact hippocampi were dissected from NKCC1$^{-/-}$ and placed in a three independent compartments chamber (Fig. 6B*a*). As for controls, kainate generated ictal-like events with γ-oscillations (Fig. 6B*a–c*) and repeated applications of kainate to one hippocampus led to the formation of a mirror focus ($n = 5/5$, Fig. 6C*a*). In further similarity to wild-type controls, interictal-like events were generated by slices prepared from the mirror focus hippocampus (Fig. 6C*b*). Therefore, this cotransporter is not necessary for the formation of a mirror focus.

Since in immature neurons, invasive recording techniques do not provide a reliable estimate of resting membrane potential (V_{rest}) (Tyzio *et al.,* 2003), we used single N-Methyl-D-aspartic acid and GABA(A) channel recordings to determine V_{rest} and the GABA driving force (DF_{GABA}), respectively, from which the reversal potential of GABA-induced currents ($E_{GABA} = DF_{GABA} + V_{rest}$) can be calculated (Fig. 7A) (Tyzio *et al.,* 2007). In control neurons, mean DF_{GABA} was positive (Fig. 7B and Supplementary Table 1). In keeping with an extensive literature (refer to 'Discussion' section), the positive DF_{GABA} was abolished by 10 μM bumetanide in NKCC1$^{-/-}$ (Fig. 7B and Supplementary Table 1) confirming that NKCC1 underlies GABA depolarization.

DF_{GABA} was significantly more depolarized in mirror focus than in control neurons (>30 mV, Fig. 7B and Supplementary Table 1) with no change of V_{rest} (−75.2 ± 5.4 mV, $n = 10$ and −78.5 ± 2.3, $n = 14$, respectively, $P = 0.08$), suggesting a persistent accumulation of chloride in epileptic neurons. In control and mirror focus neurons, DF_{GABA} was reduced by 10 μm bumetanide (Fig. 7B and Supplementary Table 1). Interestingly, bumetanide (10 μM) reduced significantly stronger DF_{GABA} in mirror focus

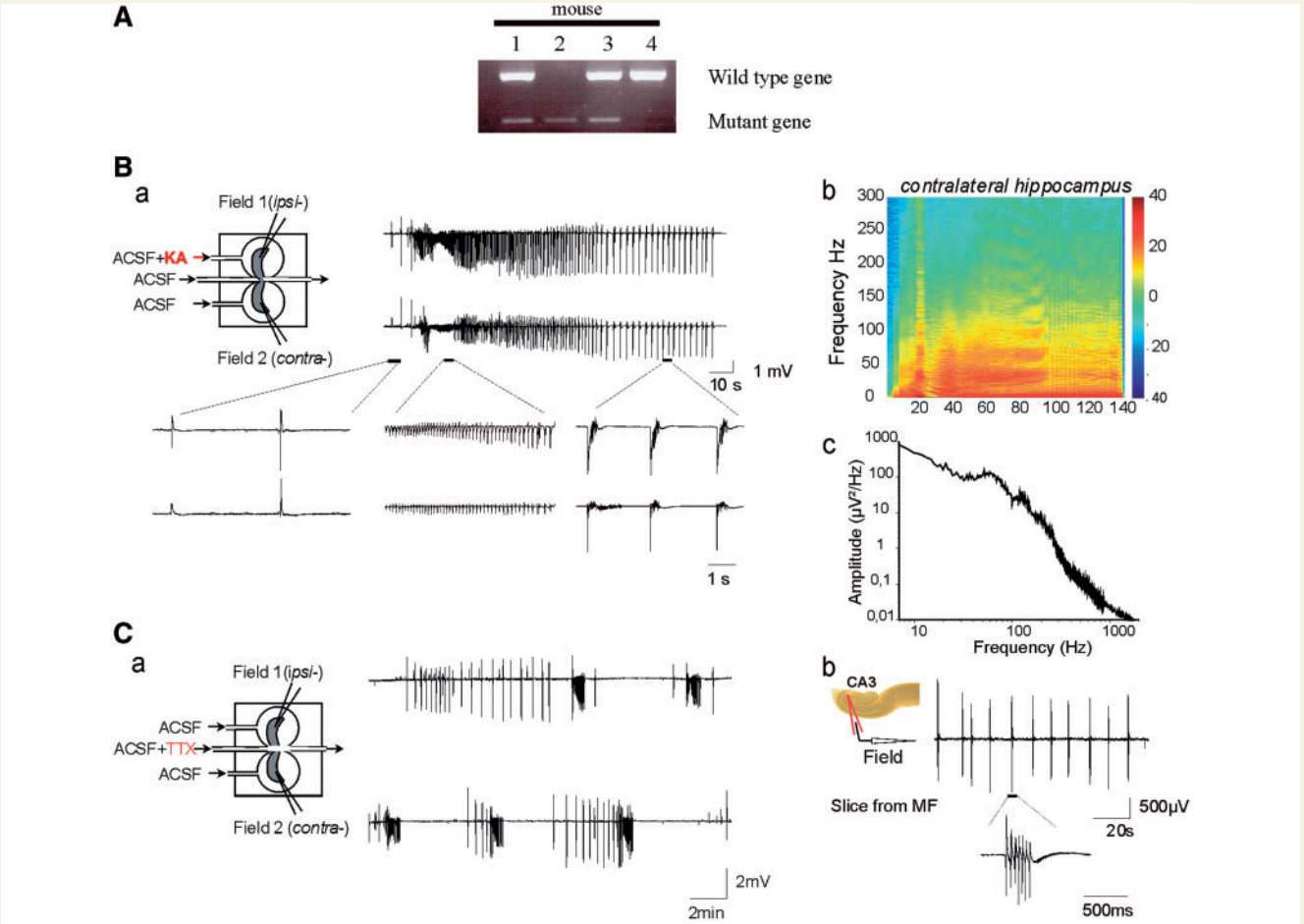

Figure 6 Genetic invalidation of NKCC1 does not prevent the formation by ictal-like events of an epileptogenic mirror focus. (**A**) Confirmation of heterozygotes NKCC1$^{+/-}$ (Mice 1 and 3) a homozygote wild-type NKCC1$^{+/+}$ (Mouse 4) and a homozygote NKCC1$^{-/-}$ (Mouse 2) by polymerase chain reaction. (**B** and **C**) Formation of a mirror focus by ictal-like events in NKCC1$^{-/-}$ mice. (**B**) Kainate was applied to one hippocampus (ipsi-) and electrical activity recorded in ipsi- and contralateral hippocampus (contra-). (**Ba**) Application of kainate to the ipsilateral side generated ictal-like events that propagated to the contralateral hippocampus. (**Bb**) γ-Oscillations in the contralateral hippocampus visualized by time-frequency wavelet analysis. (**Bc**) Power spectra of ictal-like events recorded from the contratralateral side. (**Ca**) Disconnection of the two hippocampi, after 15 unilateral applications of kainate, revealed spontaneous ictal-like events in both hippocampi. (**Cb**) Spontaneous interictal-like events generated in slices from NKCC1$^{-/-}$ mirror focus (MF) hippocampus (n = 30). ACSF = artificial cerebrospinal fluid.

than in naïve neurons (from 37.28 ± 8.08 to 14.19 ± 7.64 mV in mirror focus neurons and from 13.24 ± 12.16 to 2.56 ± 3.84 mV in control neurons, P = 0.0067; Supplementary Table 1), suggesting that the NKCC1 is operative and enhanced in epileptic neurons. However, in control neurons $E_{GABA} \approx V_{rest}$ but in both wild-type mirror focus neurons in the presence of bumetanide and in NKCC1$^{-/-}$ mirror focus neurons, E_{GABA} is significantly more positive than V_{rest} suggesting the contribution of other mechanisms.

KCC2 is downregulated in mirror focus neurons

As KCC2 knockout mice (KCC2$^{-/-}$) die at birth (Hubner *et al.* 2001), we used KCC2 antagonists to determine its role in control and mirror focus neurons. In control neurons, DF_{GABA} that was close to V_{rest} when NKCC1 was blocked selectively, shifted to highly positive values (~27–29 mV) when KCC2 was also blocked by either high concentrations of bumetanide (100 μM) that block both NKCC1 and KCC2 (Payne, 1997), or by a combined application of low concentrations of R-(+)-[(2-*n*-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy] acetic acid (DIOA; 10 μM) to block KCC2 (Pond *et al.* 2004; Boulenguez *et al.*, 2010) and bumetanide (10 μM) to also block NKCC1 (Fig. 7B and Supplementary Table 1). The importance of KCC2 is also reflected by the observation that slices incubated with DIOA (10 μM) generated interictal-like events in control slices (Supplementary Fig. 1A). In keeping with this, focal applications of GABA that generated 1.3 ± 0.4 action potentials in control neurons (n = 30), triggered bursts of action potentials (mean = 7.8 ± 1.5, n = 23, $P < 0.0001$) in DIOA treated slices (Supplementary Fig. 1B). Therefore, KCC2 is instrumental in determining DF_{GABA} in naïve neurons and when blocked GABA strongly excites neurons and interictal-like events can be generated.

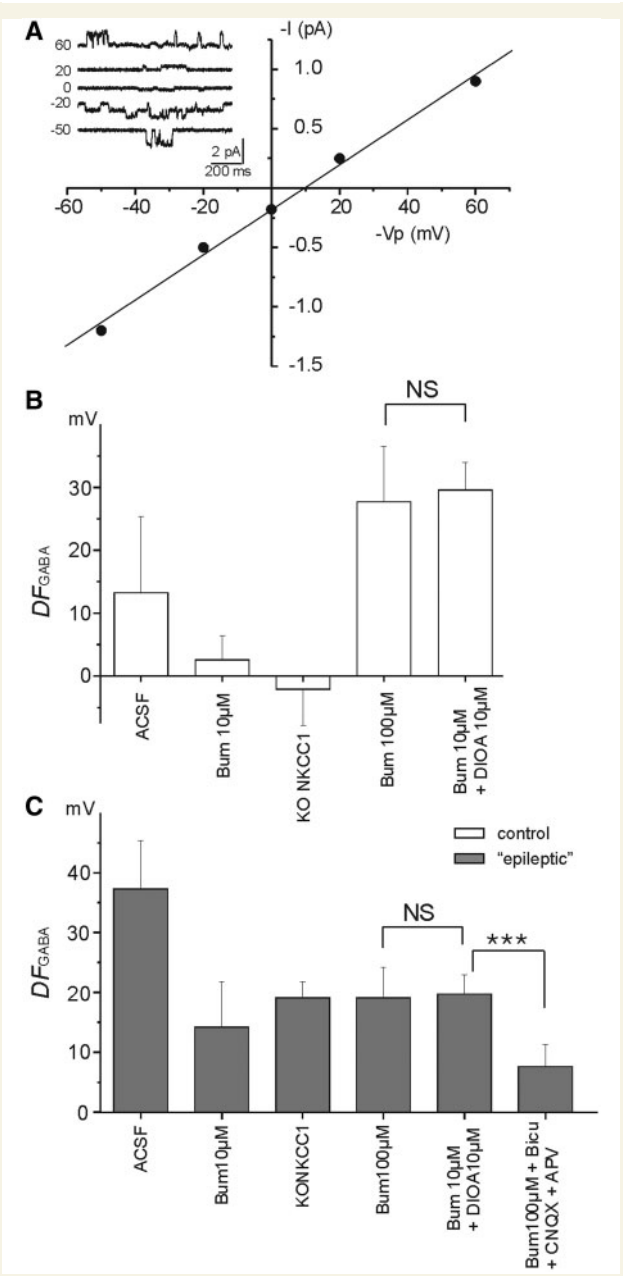

Figure 7 Preservation of NKCC1 and decreased function of KCC2 in mirror focus epileptic neurons. Single-GABA(A) channels were recorded in cell attached mode to determine the driving force (*DF*) of GABA in control and mirror focus neurons. (**A**) Single-GABA(A) channel recorded in a control neuron with the I–V curve on the right. Histograms of DF_{GABA} in control (**B**) and mirror focus (**C**) neurons in the presence of cotransporter(s) antagonists or in NKCC1$^{-/-}$ mice. See also Supplementary Fig. 1. Bum = bumetanide. ***$P < 0.001$.

Very different results were observed in mirror focus neurons. DF_{GABA} was significantly reduced in mirror focus neurons when NKCC1 was selectively blocked (from ~37 mV to 15–19 mV in the presence of bumetanide (10 µM) or in NKCC1$^{-/-}$, Supplementary Table 1) attesting to the important role of NKCC1 in mirror focus neurons. However, blocking NKCC1 and KCC2 in mirror focus neurons, by either bumetanide (100 µM) or DIOA (10 µM) and bumetanide (10 µM), produced a small difference in comparison to the DF_{GABA} values observed when NKCC1 is solely blocked (~19 mV and 14 mV, Supplementary Table 1), suggesting that KCC2 is downregulated in mirror focus neurons. Therefore, in addition to the NKCC1 activity in mirror focus neurons, there is also a loss of KCC2 operation.

Yet, DF_{GABA} remained positive after blockade of both NKCC1 and KCC2 raising the question of the underlying mechanisms. Using single-GABA channel recordings, we tested the hypothesis that ongoing augmented synaptic activity in mirror focus neurons contributes to that effect. We determined DF_{GABA} in the presence of a cocktail to block glutamatergic and GABAergic signals [6-cyano-7-nitroquinoxaline-2,3 dione (CNQX, 10 µM), D-2-amino-5-phosphopentanoate (APV, 40 µM) and bicuculline (10 µM)]. As shown in Fig. 7C and Supplementary Table 1, this considerably reduced the positive driving force suggesting that ongoing synaptic activity is instrumental in shifting DF_{GABA} in mirror focus neurons. Therefore, in addition to the enhanced NKCC1 activity and downregulation of KCC2, ongoing GABAergic and glutamatergic currents lead to chloride accumulation and enhance DF_{GABA} in mirror focus neurons.

Chloride removal is slowed down in mirror focus neurons due to downregulation of KCC2

To directly determine chloride removal from neurons, we used with some modifications a paradigm used by other authors (Zhu *et al.*, 2005; Achilles *et al.*, 2007; Brumback and Staley, 2008). In brief, neurons were recorded in the perforated patch-clamp mode to preserve intact chloride, in the presence of CNQX (10 µM) and APV (40 µM) to block ionotropic glutamate currents. The holding potential (V_H) was adjusted to have no current flow i.e. $V_H = E_{GABA}$. GABA (200 µM) was then focally pressure applied for 50–100 ms through a patch pipette to the recording neurons every 20 s (Fig. 8). Large voltage steps (to $V_H = 0$ mV, 1 min duration, three GABA pulses) were applied to depolarize neurons. Immediately after the end of the pulse, focal GABA generated inward currents suggesting that chloride has accumulated. This current was reduced progressively until it returned to pre-conditioning values ($V_H = E_{GABA}$). The latency of the time to recover (recuperation time) provides a good indication of the efficacy of KCC2 to remove chloride that has accumulated during the depolarization. Interestingly, after the return to control values, outward post-synaptic currents were recorded (Fig. 8A) suggesting a possible over activity of KCC2 (refer to 'Discussion' section).

In mirror focus neurons, recuperation time was considerably (5-fold) enhanced (from 68.4 ± 7.9 s in control to 306 ± 33.5 s in mirror focus neurons, $n = 18$ and $n = 6$, respectively, $P < 0.001$) suggesting a chronic deficiency in chloride removal. Interestingly, in contrast to naïve neurons, the transient increase of outward currents after recuperation was not observed in mirror focus neurons (not shown). Recuperation time is largely mediated by KCC2 as its specific antagonist DIOA (10 µM) or bumetanide

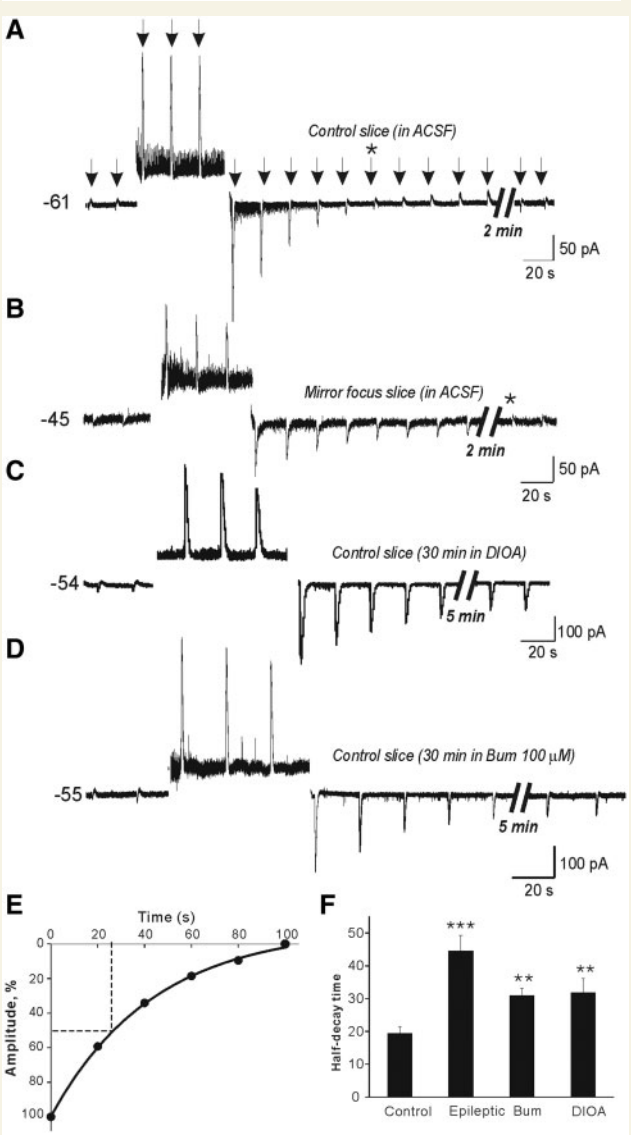

Figure 8 Dynamic removal of chloride is severely hampered in mirror focus epileptic neurons. GABA was focally applied (arrows) to evoke a response in perforated patch recorded neurons (in the presence of CNQX and APV) at a holding potential corresponding to E_{GABA}. A large depolarizing pulse from E_{GABA} to 0 mV (1 min) was applied and the delay to recovery of E_{Cl} was determined. (**A**) In a control neuron, after depolarizing pulse, GABA generated inward currents that progressively decayed to reach E_{Cl} after circa 1 min (*) (68.4 ± 7.9 s, $n = 18$). (**B**) In mirror focus epileptic neurons, the duration was increased 5-fold to ~5 min (*) (306 ± 33.5 s, $n = 5$, $P < 0.001$). (**C**) In a similar protocol, the selective blockade of KCC2 with 10 µM DIOA (**D**) or 100 µM bumetanide (Bum) dramatically augmented this delay, E_{Cl} failed to recuperate even after 10–15 min ($n = 7$ and $n = 6$, respectively). (**E**) Example of decay and half-decay time (dotted line) measurements in control neurons. The amplitude of measured currents were quantified and normalized to the amplitude of first inward currents recorded after depolarizing pulse. (**F**) Quantification histogram of half-decay times. (**$P < 0.01$, ***$P < 0.001$).

(100 µM) dramatically enhanced the half-decay time [from 19.5 ± 1.9 s in control, $n = 18$ to 31.8 ± 4.3 s in DIOA and 30.9 ± 2.2 in bumetanide (100 µM, $n = 7$ and $n = 6$, respectively, $P < 0.001$, Fig. 8)]. Recuperation time in these experiments was not quantifiable as there was no return to initial E_{GABA} even after 10 min (Fig. 8C and D). Therefore, chloride removal is altered in mirror focus neurons following a downregulation of KCC2.

Internalization of KCC2 labelling in mirror focus neurons

We used a specific KCC2 antibody that does not label neurons in KCC2$^{-/-}$ (Supplementary Fig. 2) to examine the cellular distribution of KCC2. In the rat and mice control hippocampi KCC2 was primarily located near the membrane of cell bodies and proximal processes of CA3 pyramidal neurons (Fig. 9A1–3, arrows). The labelling was often observed in clusters (Fig. 9A4, arrowheads). We observed, already at P4, clusters of KCC2 close to the cell membrane at the light (Fig. 9D1) and electron microscopy levels (Fig. 9D2–3, arrow).

In contrast, the labelling in CA3 pyramidal neurons was largely intra-cytoplasmic in mirror focus hippocampi (Fig. 9B1–3, arrowheads) with few clusters (Fig. 9B4, arrowhead), suggesting an internalization of KCC2 after repeated ictal-like events. The differences between the cellular distribution of KCC2 in naïve and mirror focus neurons (Fig. 9C) was statistically significant with a sharp peak in control hippocampi around the membrane (blue curve) and a higher and spread out labelling over the cytoplasmic compartment in mirror focus neurons (red). Therefore, there is an internalization and loss of activity of KCC2 in mirror focus neurons.

Bumetanide prevents aggravation of epileptiform activities by phenobarbital in a mirror focus

Since the NKCC1 antagonist bumetanide reduces chloride in epileptic neurons, we tested the possibility that it would also reduce the pro-epileptic actions of phenobarbital in mirror focus slices. When applied together, phenobarbital and bumetanide reduced/blocked interictal-like events generated spontaneously by mirror focus slices. However, the order of applications of the two agents revealed important differences. Applications of phenobarbital first aggravated interictal-like events (Fig. 4) that were then reduced by the additional application of bumetanide (not shown, $n = 5$). In contrast, when applied first, bumetanide strongly reduced interictal-like events that were replaced by giant depolarizing potential-like events (Supplementary Fig. 3). Addition of phenobarbital at that stage further reduced neuronal activity and giant depolarizing potential-like events ($n = 5$). Therefore, bumetanide ameliorates the effects of phenobarbital but it is preferable to apply bumetanide before phenobarbital to avoid its initial pro-epileptic actions and reinforcement of epileptiform activities.

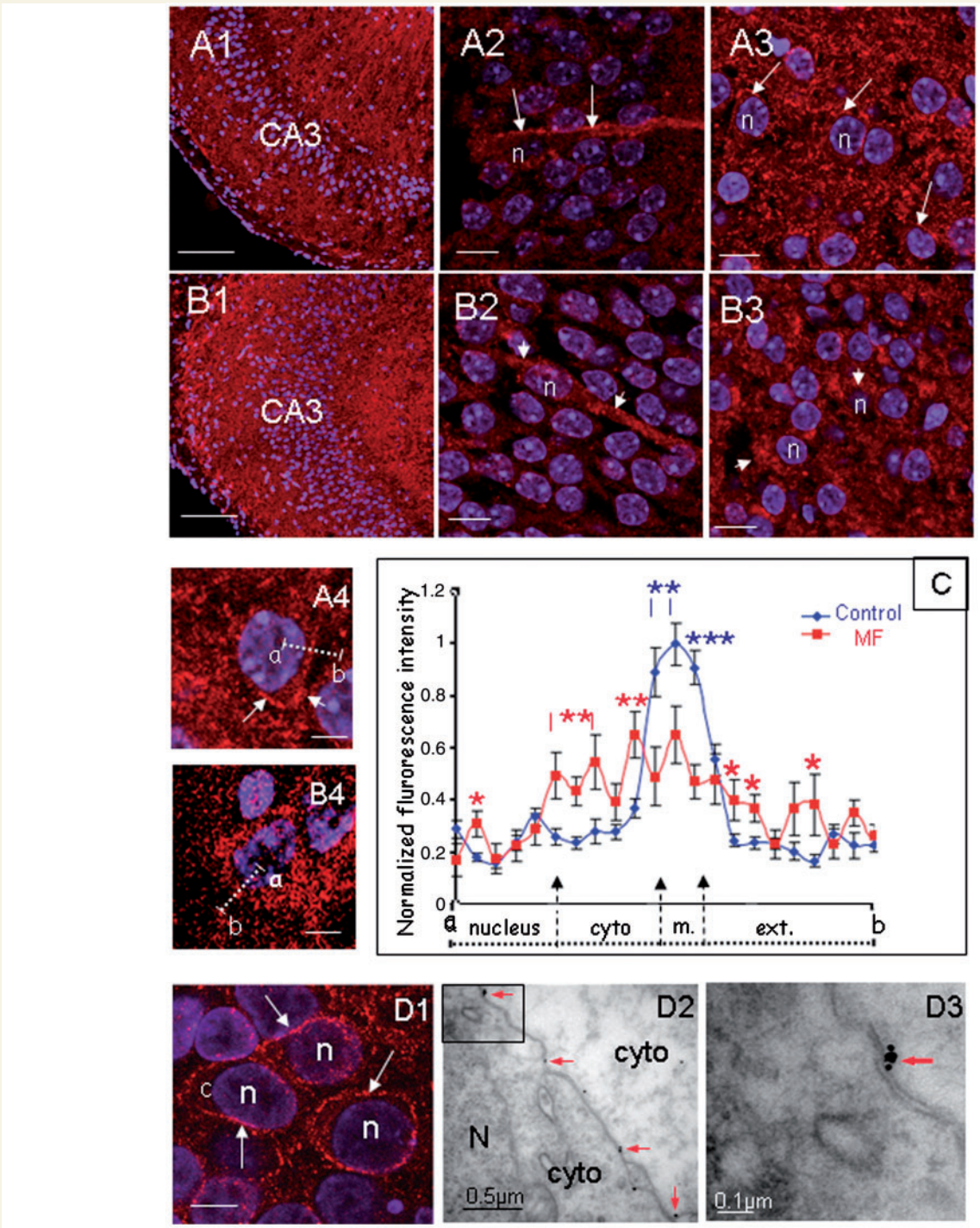

Figure 9 Changes in the subcellular localization of KCC2 immunoreactivity in control and mirror focus epileptic mice hippocampi. (**A1–3**) Control CA3 pyramidal cells. (**A1**) Low magnification shows that KCC2 is strongly expressed in CA3. The labelling closely outlines the membrane of cell bodies and processes at higher magnifications, at two levels (**A2,** near the surface; **A3,** deep in the hippocampus) (arrows). The cytoplasm is almost devoid of KCC2 labelling (**A4**, arrows). (**B1–3**) Mirror focus hippocampi. (**B1**) Low magnification shows that KCC2 is strongly expressed. However, the labelling is clearly found in the cytoplasmic cell compartments at higher magnifications of pyramidal cells located near the CA3 surface (**B2**, arrowheads) or deeper (**B3**, arrowheads). (**C**) Histograms representing the distribution and quantification of the intensity of fluorescence in 30 CA3 cells in control (blue curve) and mirror focus (MF) conditions (red curve). Fluorescence was quantified and normalized to the higher intensity. KCC2 immunoreactivity significantly increased near the cell membrane of control pyramidal cells. In contrast, the fluorescence is significantly higher in the cytoplasm compartment of mirror focus neurons. (**D1**) At P4 in control CA3 pyramidal cells, KCC2 is already located near the cell membrane (arrows). (**D2–3**): Electron-microscopic immunogold labelling showing the distribution of KCC2 near the cell membrane (arrows in **D2**). The higher magnification (**D3**) clearly shows the clustering of the gold particles near the plasma membrane (arrow). c = cytoplasm; m = membrane; N, n = nucleus; ext. = extracellular compartment. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars on histograms represent S.E.M. Scale bars: A1, B1 = 100 µm; A2, A3, B2, B3 = 10 µm; A4, B4, D1 = 2.5 µm.

Discussion

Therefore, phenobarbital reduces inaugurating ictal-like events but aggravates epileptiform activity generated by a mirror focus formed by the propagation of recurrent ictal-like events. The levels of chloride are determinant as they impose the polarity of GABAergic responses and hence the actions of phenobarbital. These changes are mediated by a parallel upregulation of NKCC1 that will import chloride more efficiently and a KCC2 downregulation and internalization that will render more difficult the export of chloride during epileptiform activities. In addition, enhanced neuronal activity in mirror focus neurons is instrumental as blocking GABA and glutamate post-synaptic currents reduce DF_{GABA} significantly. The changes persist well beyond the recurrent ictal-like events stressing the importance of the history of seizures before phenobarbital injection that downregulate KCC2. In that respect, early treatment with the diuretic bumetanide may help preventing this cascade and preventing KCC2 loss and the shift of polarity of GABA actions and the paradoxical effects of phenobarbital.

Recurrent ictal-like events enhance GABA excitatory drive that are exacerbated by phenobarbital

Although time consuming, the single-GABA(A) and N-Methyl-D-aspartic acid channel recordings used to determine chloride DF_{GABA} and V_{rest}, respectively, is the only method that provides precise determination of these values in immature neurons with a major shift of V_{rest} in all invasive recordings including perforated patch recordings (Tyzio *et al.,* 2003, 2008). DF_{GABA} is enhanced in mirror focus neurons and the number of spikes generated by GABAergic synapses is increased. GABA excites epileptic neurons in many animal models of epilepsy (Khalilov *et al.,* 2003, 2005; Rivera *et al.,* 2005; Huberfeld *et al.,* 2007; Kahle *et al.,* 2008; Li *et al.,* 2008) and human epileptic neurons (Cohen *et al.,* 2002; Cepeda *et al.,* 2007; Huberfeld *et al.,* 2007; Andre *et al.,* 2008; Tyzio *et al.,* 2009).The converging actions of GABA depolarization and voltage gated currents or activation of NMDA receptor-mediated excitatory post-synaptic currents (Sipila *et al.,* 2006; Ben-Ari *et al.,* 2007; Valeeva *et al.,* 2010) will efficiently alter brain network operation.

The polarity of GABA actions is altered by even brief coincident activation of pre- and post-synaptic sites (Woodin *et al.,* 2003; Yamada *et al.,* 2004; Achilles *et al*., 2007). Suggested mechanisms include pre-synaptic reduction of GABA release on dendrites of target neurons (Hirsch *et al.,* 1999), trafficking, intracellular accumulation of GABA receptors and internalization of GABA receptor subunits (Loscher and Honack, 1989; Mikati *et al.,* 1994; Brown-Croyts *et al.,* 2000; Goodkin *et al.,* 2005, 2007, 2008; Naylor *et al.,* 2005; Goodkin and Kapur, 2009). The potent actions of phenobarbital on mirror focus neurons suggest that phenobarbital recognition sites are not inactivated in the mirror focus.

NKCC1 activity and KCC2 downregulation in mirror focus neurons

A plethora of mechanisms control intracellular chloride and could determine the shifts observed in epileptic neurons (Staley, 1994; Staley *et al.,* 1996; Payne *et al.,* 2003; Hentschke *et al.,* 2006; Brumback and Staley, 2008; Jacobs *et al.,* 2008*b*; Zhu *et al.,* 2008; Pfeffer *et al.,* 2009; Dzhala *et al.,* 2010; Foldy *et al.,* 2010). Several studies, in particular by Staley *et al.,* (1996), focused on NKCC1 showing that it is upregulated in epileptic neurons, providing an explanation to the potent actions of bumetanide on epileptic activity (Dzhala *et al.,* 2005, 2008; Kilb *et al.,* 2007; Nardou *et al.,* 2009). Present observations confirm these earlier studies. However, continuous perfusion with bumetanide in the triple chamber (Nardou *et al.,* 2009) or genetic invalidation of NKCC1 (the present report) neither prevented the formation of a mirror focus nor the alterations of DF_{GABA} suggesting that this cotransporter is not mandatory for these changes to occur and that other mechanisms must operate. Interestingly, bumetanide is more efficient in some seizures than in others (Kilb *et al.,* 2007) and bumetanide is not efficient in adult *in vivo* models of temporal lobe epilepsies (Brandt *et al.,* 2010). Therefore, it is safe to conclude that NKCC1 is indeed upregulated in some, but not all types of epilepsies, and that other mechanisms are instrumental.

Here we suggest that KCC2 is downregulated in mirror focus neurons. Indeed, KCC2 has been shown to play an important role in chloride extrusion in maturation, neuronal excitability and seizures (Lu *et al.,* 1999; Rivera *et al.,* 1999; Ganguly *et al.,* 2001; Hubner, *et al.,* 2001; Owens and Kriegstein, 2002; Gulacsi *et al.,* 2003; Chudotvorova *et al.,* 2005; Zhu *et al.,* 2005; Blaesse *et al.,* 2006, 2009; Ben-Ari *et al.,* 2007; Wang and Kriegstein, 2008, 2009). KCC2 is localized on the membrane of many immature CA3 pyramidal neurons (Gulyas *et al.,* 2001), it has a high turnover, its distribution correlates with GABAergic inhibition (Gulacsi *et al.,* 2003; Szabadics *et al.,* 2006) and once present on the membrane becomes operative after a process of dimerization that correlates with the development of inhibitory transmission (Blaesse *et al.,* 2006). Here, we show that KCC2 antagonists dramatically augment DF_{GABA} in control neurons, slow down the recuperation after a chloride loading stimulus and even generate epileptiform activities attesting to its important roles. Although other interpretations of our measures of chloride removal are possible (Brumback and Staley, 2008), several observations suggest that these observations are mediated by KCC2. In keeping with the upregulation of KCC2 after single seizures (Khirug *et al.*, 2010), we observed that GABA induced post-synaptic currents are transiently outward in the chloride loading experiments (Fig. 8A) reflecting an over activity following a single seizure. These outward currents are neither observed in the presence of KCC2 antagonists (Fig. 8C and D) nor in mirror focus neurons (Fig. 8B). Also, in control neurons, DF_{GABA} is reduced by a selective blockade of NKCC1 but enhanced by a selective blockade of NKCC1 and KCC2 (to depolarizing values). In contrast, blocking only NKCC1 or both NKCC1 and KCC2 in mirror focus neurons produced similar DF_{GABA} values suggesting that in epileptic neurons KCC2 is downregulated.

KCC2 has been shown to be downregulated by a wide range of insults including spinal cord transections, traumatic insults, brain lesions and seizures (Coull *et al.,* 2003; Payne *et al.,* 2003; Price *et al.,* 2005; Huberfeld *et al.,* 2007). KCC2 is internalized after seizures (Papp *et al.,* 2008). Several observations suggest a high degree of turnover and internalisation of KCC2 after various insults. KCC2 cell surface stability and activity are controlled by tyrosine phosphorylation (Lee *et al.,* 2007, 2010; Robinson *et al.,* 2010). Recently, Lee and colleagues (Lee *et al*., 2010) have identified the sites of tyrosine phosphorylation within KCC2 residues and shown that phosphorylation of these sites decreases the cell surface stability of KCC2 by enhancing lysozomal degradation. Status epilepticus in mice produces both tyrosine phosphorylation of KCC2 and internalisation of the cotransporter providing direct evidence on the lability of KCC2 and its internalisation after seizures (Rivera *et al.,* 2004; Wake *et al.,* 2007; Lee *et al.,* 2010).

Whether the two recently identified KCC2 isomers contribute differently to neonatal seizures (Uvarov *et al.,* 2007) and whether this is due to tyrosine phosphorylation remains to be investigated.

Here, we show that DF_{GABA} that remained positive after a block of NKCC1 and KCC2, shifted to values close to V_{rest} in mirror focus neurons when GABAergic signals are blocked. This suggests that the enhanced GABAergic activity in epileptic neurons contributes to the elevated intracellular chloride levels. Therefore, ictal-like events augment ongoing GABAergic activity and intracellular chloride leading to an important chloride influx that will not be extruded between events because of the downregulation of KCC2. The first effect of phenobarbital is to augment the severity of ictal-like events in an epileptic network. This initial aggravation may contribute to downregulate and internalize KCC2 suggesting that it may be important to avoid this as much as possible. Bumetanide prevented this initial aggravation of epileptiform activities by phenobarbital suggesting that combined use of bumetanide and phenobarbital is a promising therapeutic strategy (Dzhala *et al.,* 2008).

Clinical implications

The aggravation of neonatal seizures by anti-epileptic drugs is a complex issue with several possible mechanisms. Certain types of epilepsies are exacerbated from the onset of treatment, i.e. West syndrome by carbamazepine (Talwar *et al.,* 1994) absence and myoclonic epilepsies by vigabatrin (Lortie *et al.* 1993) or Dravet syndrome by lamotrigine (Guerrini *et al.,* 1998); others, notably GABA acting anti-epileptic drugs, lose their efficacy or even aggravate seizures after repeated seizures, i.e. diazepam (Knudsen 1979; Goodkin and Kapur, 2009) or pentobarbital (Rantala *et al.,* 1999; Mikaeloff *et al.,* 2006; Chipaux *et al.,* 2010).

Here, we used *in vitro* experiments to gain information on these issues. These types of experiments have intrinsic limitations when compared to the clinical situation because of the lack of major cerebral connections, the lack of major systemic events (vascular, hormonal, etc.) and the different species for example. Nevertheless, the triple chamber used here has several advantages over other *in vitro* preparations (slices or intact preparations). First, as the convulsive agent is applied only to one hippocampus and the alterations of GABA and phenobarbital effects investigated in the other, there are no interactions between these alterations and the convulsive agents/conditions used to generate them (e.g. Mg^{2+}, bicuculline and kainate). Secondly, the effects of phenobarbital are determined either on naïve networks and/or a mirror focus formed by recurrent propagated ictal-like events. We show that phenobarbital blocks initial ictal-like events but aggravates epileptiform activities generated by epileptic neurons in a mirror focus, suggesting that GABA acting anti-epileptic drugs may depend on the history of seizures prior to treatment. The mechanism underlying these changes in phenobarbital actions differs from previously reported loss in GABA-acting drugs efficacy due to internalization of GABA(A) receptors (Goodkin and Kapur, 2009) and involves change in the intracellular chloride homeostasis and associated shifts in the driving force for GABA(A)-mediated currents. Predictions from our study that maybe of interest to explore in the clinical studies are that: (i) phenobarbital is efficient for treating early seizure but its efficiency reduces or even inverts when phenobarbital is applied following multiple recurrent seizures; (ii) in keeping with our previous observations that high frequency (in γ-range, 40–120 Hz) oscillations are the hallmark of the epileptogenic process and phenobarbital efficiently suppresses γ-oscillations and prevents epileptogenic processes if administered early on. Its timely administration, which can be assayed by the efficiency in the epileptic focus, may prevent secondary epileptogenesis; and (iii) anticonvulsive phenobarbital efficacy may be reinstated even at the late phases of secondary epileptogenesis by combination with drugs that reinstate inhibitory action of GABA, such as NKCC1 antagonist bumetanide. An early intervention with the diuretic NKCC1 antagonist is strongly suggested in order to preserve KCC2, reduce as much as possible the excitatory actions of GABA and therefore reinforce the anti-epileptic actions of phenobarbital. This is in keeping with Dzhala *et al.* (2008), who first showed the advantages of this anti-convulsant polypharmacy. However, further investigations on a protection of KCC2 from downregulation and internalization by seizures may provide novel therapeutic perspectives.

Acknowledgements

We are grateful to Dr C. Hubner for NKCC1$^{-/-}$ mice. Since the submission of this article, Dzhala *et al.* (2010) have reported that the progressive accumulation of chloride is mediated by upregulation of NKCC1.

Funding

This work was supported by the French Medical Research council (INSERM), the Université de la Méditerranée, the French agency of research ANR (L'Agence Nationale de la Recherche) (to I.Kh.), Fondation pour la Recherche Médicale (FRM) (to R.N.). The European Commission 7th Framework Program (Project NEMO) but an ERC senior neuroscience program was rejected (out of interest). YBA is in receipt of an interface contract between INSERM and the University of Paris.

Supplementary material

Supplementary material is available at *Brain* online.

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