



Antiepileptic effects of endogenous beta-hydroxybutyrate in suckling infant rats

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Received 6 October 2010; received in revised form 28 February 2011; accepted 6 March 2011

Available online 5 April 2011

KEYWORDS

Neonate;
Ketogenic diet;
Seizure;
GABA

Summary Physiological ketosis is a hallmark of metabolism in suckling infants. However, little is known on the impact of physiological ketosis on brain excitability. We addressed this question in suckling rats *in vivo*. 16-channel extracellular field potential recordings were performed from somatosensory barrel cortex at postnatal days 5–9 non-anaesthetized rat pups. Seizures were induced by the volatile convulsant agent flurothyl. One hour after blockade of physiological ketogenesis using combined administration of beta-oxidation inhibitors mercaptoacetate, insulin and glucose to prevent hypoglycemia, we found no significant change in the flurothyl-induced electrographic seizures. However, build-up of seizures during two repetitive flurothyl applications was strongly aggravated in the animals with blocked ketogenesis. The effect of ketogenesis inhibitors was reversed by exogenous beta-hydroxybutyrate. Diazepam exerted anticonvulsive action both under physiological ketosis and after blockade of ketogenesis, and bumetanide had no significant anticonvulsive effects in both conditions. Thus, physiological ketosis reduces excitability in the immature brain and elimination of physiological ketosis results in elimination of this anticonvulsant effect. Our study raises concern that the changes in diet, and pharmacological manipulations such as glucose infusion, and pathologies such as hyperinsulinism which break natural ketosis, may be a potential risk factor for epileptogenesis in nursing infants.

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Introduction

The ketogenic diet (KD) is a high-fat, low-carbohydrate, and low-protein diet widely used to treat epilepsy in children (Bough and Rho, 2007; Freeman et al., 2006; Hartman et al., 2007; Neal et al., 2008; Prasad et al., 1996; Rubenstein,

2008; Vining et al., 1998). The KD is characterized by increased fatty acid oxidation in the liver and production of ketone bodies, which along with glucose serve as an energy fuel for the brain during early developmental (Bougnères et al., 1986; Ferre et al., 1978, 1986; Nehlig, 1999). The KD has been shown to be effective in a number of animal epilepsy models (Bough and Rho, 2007; Hartman et al., 2007; Hori et al., 1997; Martillotti et al., 2006; Muller-Schwarze et al., 1999; Rho et al., 1999, 2002; Todorova et al., 2000).

While the KD purposefully produces ketosis through dietary means several physiological states are also associ-

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ated with ketosis. Most interestingly from a developmental viewpoint, feeding with maternal milk provides conditions for physiological ketogenesis, which occurs due to elevated milk fat content (Ferre et al., 1978; Nehlig, 1999). Under physiological conditions, plasma ketone bodies level in suckling infants attain values close to those attained with the KD (Bougnères et al., 1986; Ferre et al., 1986). Physiological ketosis in suckling infants is characteristic of other mammalian species, including rodents, suggesting that it is a universal feature of immature metabolism aimed to meet high metabolic demand of the developing brain (Ferre et al., 1978; Nehlig, 1999). However, the impact physiological ketogenesis has on brain excitability at present is unknown. This question is of major clinical importance because, on one hand, the immature brain is more prone to seizures than the adult brain but on the other hand, the period of enhanced excitability overlaps with the suckling period (Holmes et al., 2002). Indeed, the incidence of seizures is highest during the first postnatal months with a dramatic fall after the first year of life (Forsgren et al., 2005; Hauser, 1992; Hauser et al., 1993). Moreover, clinical observations in patients on the KD indicate that the reversal of seizure protection when ketosis ends can be nearly immediate—after eating a candy bar or upon IV infusion of glucose, seizure activity in patients prone to frequent seizures can recur within hours (Huttenlocher, 1976; Rubenstein, 2008). This raises the hypothesis that physiological ketosis acts as a natural anticonvulsive mechanism in suckling infants, and that ending ketosis would facilitate seizure and the epileptogenic process. We addressed these questions in the suckling rat pups using the flurothyl model of seizures, and show that physiological ketosis indeed modulates brain excitability. Although blockade of ketogenesis did not significantly modify seizures evoked by single exposure to flurothyl, it significantly aggravated seizure build-up upon repetitive flurothyl exposures. Our observations raise a concern that changes in diet and pharmacological manipulations which eliminate natural ketosis are a potential risk factor for epileptogenesis in nursing infants.

Materials and methods

This study followed INSERM guidelines on animal care, with approval from the animal care and use local committee. Wistar rats of both sexes from postnatal days [P] 5–9 were used (P0 = day of birth). According to comparative studies in human and rats, the level of maturity in this age group roughly corresponds to the human newborn (Avishai-Eliner et al., 2002; Clancy et al., 2001; Dobbing and Sands, 1979; Gottlieb et al., 1977). Intracortical EEG recordings were performed from head restrained non-anaesthetized rat pups as described previously (Khazipov et al., 2004b; Minlebaev et al., 2007). In brief, during the surgical procedure, which lasted for 15–20 min, rats were anesthetized with isoflurane (1.5%) using Fluotec4 (Surgivet/Anesco) anesthesia apparatus. The skin and periosteum were removed from the skull, which was then covered with a layer of dental acrylic, except an area ~1 mm in diameter above the barrel cortex. Nuchal muscles were cut from the skull to reduce movement artifacts. Following 1–2 h recovery period, the rat was positioned in the stereotaxic apparatus, and the skull was attached to the nose (nasal bones) and ear bars (occipital bone) with dental acrylic (Fig. 1A). During recordings, rats were placed at heated platform (37°C) using an automatic temperature controller (TC-344B; Warner Instruments, Hamden, CT). Extracellular

field potential recordings were performed using 16 channel silicon probes with 100 μ m separation distance between the channels (Neuronexus technologies, MI) inserted vertically into the cortex (Fig. 1C). Signals were amplified (1000 $\times$, bandpass 1 Hz to 5 kHz) using custom built amplifiers (A. Alexeev, Trinity, Russia), digitized at 10 kHz using Digidata 1440 interface (Axon Instruments) and analyzed offline using an Axon package (Axon Instruments) and Matlab (MathWorks, Natick, MA).

Seizures were induced by inhalation of flurothyl (0.1 ml) added to a mask-chamber of 1.5 ml volume, with 90 s exposure duration. In the paired flurothyl exposure experiments, the animals were exposed to flurothyl twice with a 2 h interval. Electrographic seizure analysis was performed as following. EEGs were first converted to Matlab files. Multiple units were detected after high-pass filtering (>200 Hz) with a spike threshold set at 4 standard deviations from baseline. Wide-band EEG signal was then downsampled at 1 kHz for the local field potential analysis. For the current source density (CSD) analysis, threshold for detection of population spikes in L2/3 was set at -200μ V. CSD was computed for each recording site according to differential scheme for second derivative and smoothed with a triangular kernel (Freeman and Nicholson, 1975).

To quantify seizure, we calculated scalar seizure integral (Σ) at the layer 2/3 recording site in which was located the major sink of the epileptiform activity. Σ is a sum of the local field potentials (both sinks and sources) comprising seizure. Σ was calculated as following: (i) raw EEG signal in the L2/3 was referenced to the most superficial recording site located at the cortical surface to abolish movement artifacts and volume conducted signals from adjacent areas and converted to scalar EEG by inverting all negative values to positive values; (ii) scalar EEG was corrected for the baseline activity level by subtraction of the mean scalar EEG value calculated during 5 min before flurothyl exposure; (iii) scalar seizure integral (Σ) was calculated as cumulative, corrected for the baseline, scalar EEG during 10 min after flurothyl exposure. In the paired flurothyl exposure experiments, Σ of the second seizure was normalized to the first response.

Glucose levels were measured in mixed arterial/venous 200 μ l blood samples obtained after decapitation using Accu-Check Performa glucometer (Roche, Mannheim, Germany).

Drugs were administered as following: bumetanide (50 mg/ml in DMSO diluted to 0.5 mg/ml with 0.9% NaCl) was applied intraperitoneally (i.p.) at a dose of 5 μ mol/kg. Insulin (0.07 IU/ml diluted in 0.9% NaCl) was applied intramuscularly (i.m.) at a dose of 0.33 IU/kg. Glucose (0.4 mg/ml diluted in 0.9% NaCl) was applied at 5 mmol/kg (i.p.). 2-Mercaptoacetate (40 mg/ml diluted in 0.9% NaCl) was administered at 100 mg/kg (i.p.). DL-BHB (100 mg/ml diluted in 0.9% NaCl) was applied at 2 and 4 mmol/kg (i.p.) that corresponds to 1 and 2 mM of D-BHB, respectively. Diazepam (1 mg/ml diluted in 0.9% NaCl) was administered at 5 mg/kg (i.p.). Drugs were purchased from Fluka (DL-beta-hydroxybutyrate), and all other compounds from Sigma (St. Louis, MO).

Group measures are expressed as means $\pm$ 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

We used 16-channel silicone electrodes to study the epileptiform activity induced by flurothyl in the barrel somatosensory of one week old rat pups (postnatal days [P] 5–9). Prior to flurothyl exposure, the electrographic activity was discontinuous with intermittent sharp potentials and spindle-bursts characteristic of this developmental stage (Khazipov et al., 2004b; Minlebaev et al., 2007). In the present study, we used brief, high dosage exposure to flurothyl (0.1 ml added to the 1.5 ml mask, 90 s exposure

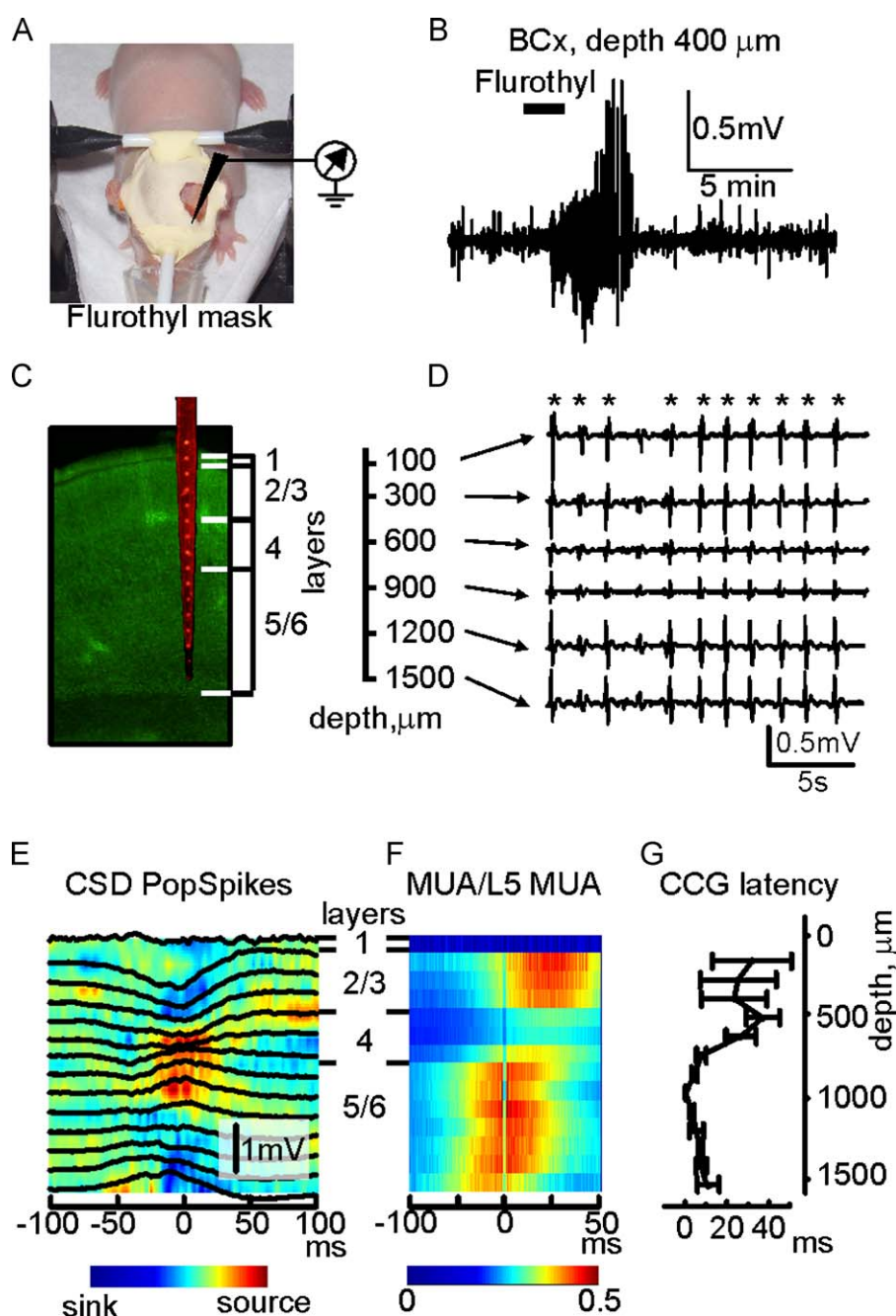


Figure 1 Flurothyl-evoked neocortical seizures in the rat pups. (A) Experimental setup of 16-channel silicone probe recordings (electrode is shown on panel C) from the head-restrained, non-anesthetized rat pup. (B) Electrographic seizure in layer 2/3 barrel cortex evoked by flurothyl inhalation. (C) Microphotograph of a cresyl labeled coronal section of barrel cortex with a recording 16 channel silicone probe overlaid on a Dil stained track. (D) Expanded trace of the flurothyl-evoked seizure at different cortical depths with population spikes marked by asterisks. (E) Averaged population spikes at different cortical depths (black traces) triggered by a peak of population spikes in layer 2/3 (electrode #4) overlaid on the corresponding CSD; $n = 43$ population spikes. (F) Crosscorrelogram of MUA in different cortical layers versus layer 5 MUA (total 10,832 spikes). (B–F): P7 rat. (G) MUA delays at different cortical depths in relation to layer 5 (depth 900 μ m) MUA. Note that population spikes originate from layer 5 and spread through the entire column. (G) Pooled data from eight P5–8 rats.

duration). In agreement with the results of previous studies in young rats, exposure to flurothyl readily evoked motor and electrographic seizure (Holmes et al., 1998; Huang et al., 1999; Okada et al., 1986; Rho et al., 1999; Sperber et al., 1999; Sperber and Moshe, 1988). Seizure occurred at a

30–60 s delay after the beginning of flurothyl exposure and lasted for 3–5 min. The onset of electrographic seizure was characterized by emergence of population spikes occasionally intermingled with high frequency oscillations (30–60 Hz) which lasted for 5–10 s (Fig. 1). Population spikes regu-

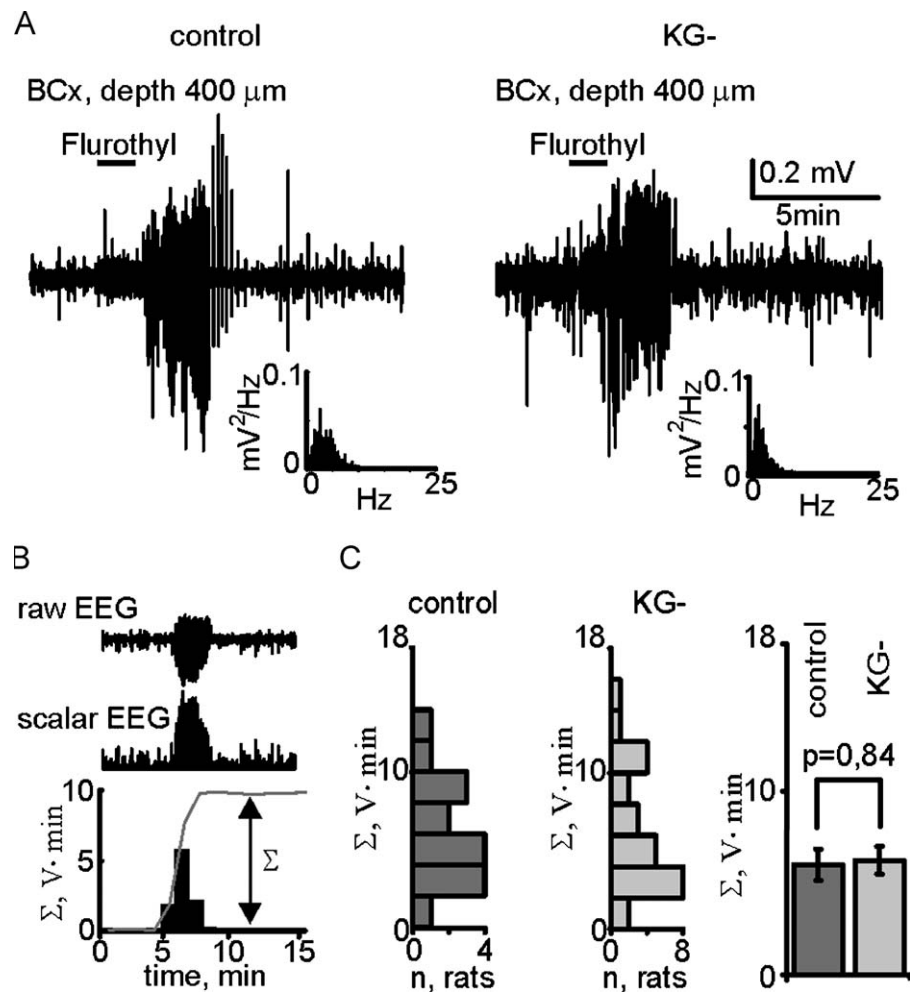


Figure 2 Blockade of ketogenesis does not affect single flurothyl-evoked seizure. (A) Extracellular field potential recordings of the flurothyl-evoked seizures from layer 2/3 barrel cortex in control animal with ketogenesis intact (left trace) and 1 h after blockade of ketogenesis (KG-) by combined administration of mercaptoacetate and insulin (with glucose to prevent hypoglycemia). Corresponding power spectrum of seizures is shown on insets. (B) Algorithm for seizure quantification. Raw EEG is converted to scalar EEG, corrected for baseline activity level during 5 min before flurothyl exposure, and residual scalar seizure integral (Σ) is calculated. (C) Histogram plots of scalar seizure integrals distribution in control conditions (left, $n = 16$ P5–8 rats) and after blockade of ketogenesis (middle, $n = 26$ P5–8 rats), and pooled data average (right, means $\pm$ S.E.). Note that blockade of ketogenesis does not significantly modify single flurothyl-evoked seizure.

larly occurred at $0.5s^{-1}$ at the beginning of seizure and their frequency progressively decreased to $0.1s^{-1}$. Analysis of the current source density and multiple unit activity associated with epileptic population spikes was performed in eight P5–8 animals. Current source density analysis of population spikes revealed two sinks located in infragranular and supragranular cortical layers and a source in the granular layer. The maximal amplitude of population spikes of $0.86 \pm 0.15 mV$ was attained in layers 2/3 at the depth $387 \pm 40 \mu m$ (P5–8; $n = 8$) (Fig. 1E). Multiple unit activity (MUA) analysis revealed that columnar activation typically started at the depth of $875 \pm 90 \mu m$ (Fig. 1F) that corresponds to the layer 5 in the barrel cortex at this age (Fig. 1C) and further spread to the layer 6 and superficial layers (Fig. 1F and G). These results suggest that the flurothyl-induced epileptiform activity is initiated in the layer 5, keeping with the higher intrinsic excitability of layer 5 neurons (Rheims et al., 2008). Relatively long delays and weaker

recruitment of layer 4 units suggest that seizures are primarily generated by intracortical circuits with participation of the cortico–thalamo–cortical loop.

In order to quantify electrographic seizure activity, we calculated scalar integrals of the local field potential associated with seizure in the layers 2/3, where the amplitude of population spikes was maximal (Fig. 2B). Scalar seizure integral (Σ) was calculated as a sum of both negative and positive deflections of the local field potential (see Methods for details). The scalar integral of the flurothyl induced electrographic seizure (Σ) in the control rat pups was of $6 \pm 0.8 V \cdot min$, varying from 1.7 to 12.6 $V \cdot min$ (Fig. 2C; P5–8; $n = 16$).

We next studied how blockade of natural ketogenesis (KG) affects flurothyl-induced seizures. KG was blocked by administration of insulin, an inhibitor of fatty acid oxidation ($0.33 U/kg$ i.m. injected along with glucose ($5 mmol/kg$, i.m.) to prevent hypoglycemia) (Fukao et al., 2004; Storer

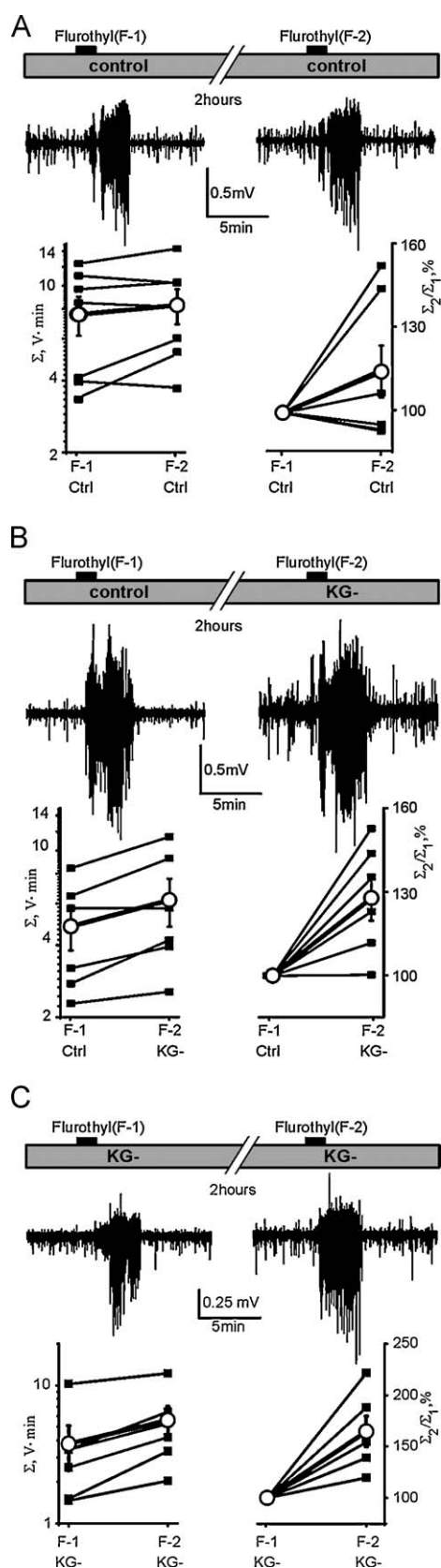


Figure 3 Blockade of natural ketogenesis aggravates seizure buildup during repetitive flurothyl exposure. (A) Two consecutive exposures to flurothyl were performed at 2 h interval in the control animal with intact ketogenesis. Traces show the local

et al., 1980) in combination with 2-mercaptoacetate (MA), a mitochondrial acyl-CoA dehydrogenases inhibitor (100 mg/kg, i.p.) (Boutellier et al., 1999). As previously reported, these treatments reduced plasma levels of the principal ketone metabolite 3-beta-hydroxybutyrate (BHB) from 0.91 to 0.14 mM measured 1 h after injections (Tyzio et al., 2011). As a result of glucose administration, glucose plasma levels slightly increased from 6.22 ± 0.31 to 8.88 ± 0.73 mM (P7; $n = 27$). Under conditions of blocked KG, flurothyl evoked seizures with an electrographic phenotype similar to that observed in the control group (Fig. 2A). Group data analysis revealed no significant difference in the distribution and mean values of the scalar seizure integrals between the control and KG-blocked animals (Fig. 2C). These results indicate that single seizure evoked by flurothyl in the rat pup neocortex is not influenced by natural ketosis.

Seizure aggravation during repetitive exposures to pro-convulsive agents or conditions, also known as “kindling” phenomenon, is a hallmark of the epileptogenic process (Gowers, 1901; Khalilov et al., 2003, 2005). Therefore we next studied how physiological KG modulates this phenomenon using paired seizure provocation, in which two standard flurothyl exposures were performed with a 2 h interval and scalar integrals of the first and second seizures were compared. In the control group, both aggravation ($n = 4$ of 7 rats) and alleviation ($n = 3$ of 7 rats) of the second seizure were observed (Fig. 3A; P6–8; $n = 7$). On average, there was a tendency to second seizure aggravation manifested by increase of the scalar seizure integrals from 7.7 ± 1.4 V min to 8.4 ± 1.4 V min, but this augmentation was not significant ($114.8 \pm 9.3\%$; Fig. 6A; $p = 0.15$). Blockade of KGs significantly increased second seizure aggravation, as revealed by two types of experiments. In the first experiment, first seizure was induced by flurothyl in the control conditions with intact KGs, following 1 h KG was blocked by combined administration of insulin and mercaptoacetate as described above, and after another hour the second exposure to flurothyl was performed (Fig. 3B). The second seizure in this protocol was more robust than the first one in five of 6 animals. On average, consecutive seizure aggravation attained $127.9 \pm 8.1\%$ (Fig. 3B; P6–7; $n = 6$; $p = 0.04$). In the second experiment, KG was blocked by insulin and mercaptoacetate administered 1 h prior to the first and second flurothyl exposures, so that both flurothyl exposures were performed in conditions of blocked KG. Under these conditions, consecutive seizure aggravation increased to $164.7 \pm 14.8\%$ (Fig. 3C; P5–8; $n = 6$; $p = 0.002$). Taken together, these experiments

layer 2/3 field potential response to the first (F-1) and second (F-2) flurothyl exposures. Group data below show: *left*, first and second seizure integrals in individual animals (black dots) and means $\pm$ S.E. (open circles), and *right*, corresponding ratio of scalar integrals of the first and second seizures ($\Sigma_2/\Sigma_1, \%$). Note variability in the consecutive seizures ratio with an average tendency to aggravation (non-significant; P6–8, $n = 7$). (B and C) Responses to repetitive flurothyl exposure performed after blockade of ketogenesis 1 h before the second flurothyl exposure (B, P6–7, $n = 6$) and before the first and second flurothyl exposures (C, P5–8, $n = 6$). Note that blockade of physiological ketogenesis aggravates consecutive seizure.

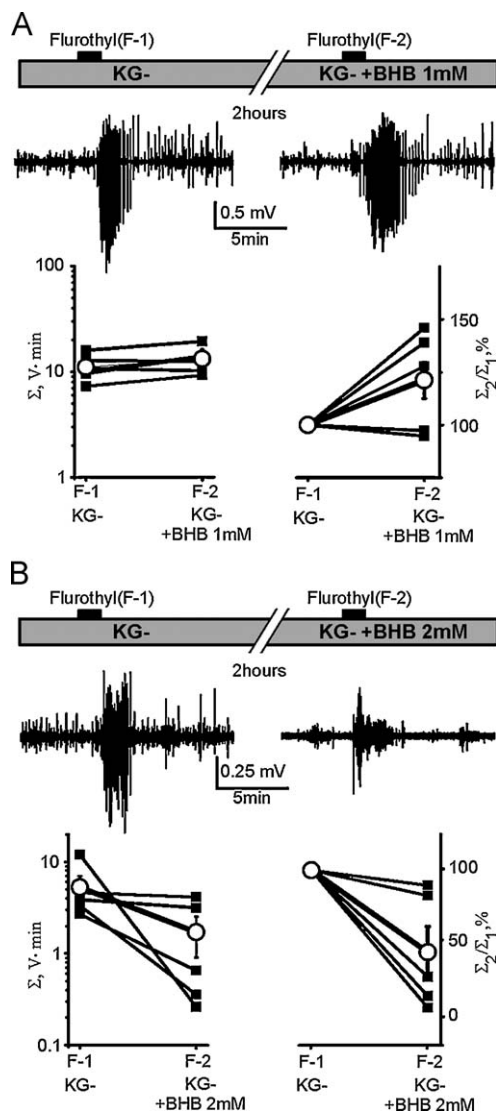


Figure 4 Effects of exogenous beta-hydroxybutyrate on consecutive seizures. (A and B) In the conditions of blocked ketogenesis, exogenous beta-hydroxybutyrate (BHB) was administered 1 h before the second exposure to flurothyl to restore the physiological level of BHB (A, 1 mmol/kg, P6–9, $n=6$) and at higher concentration (B, 2 mmol/kg, P6–8, $n=5$). Note a reduction in aggravation of consecutive seizures after administration of BHB at physiological concentrations (A) and reversal of aggravation to alleviation of consecutive seizures at elevated concentration of BHB (B).

suggest that blockade of natural KG rapidly and strongly potentiates seizure build up during recurrent seizures.

Because reduction in the ketone bodies levels is the principal direct consequence of blockade of KG, we hypothesized that restoration of ketone bodies levels should reverse the effects produced by KG blockade. Natural ketosis in this experiment was blocked by insulin and mercaptoacetate administered 1 h before the first and second flurothyl exposures. BHB was administered intraperitoneally (1 mmol/kg) 1 h before the second flurothyl exposure. As a result, consecutive seizure aggravation was reduced to $121.1 \pm 8.6\%$ (Fig. 4A; P6–9; $n=6$), that is significantly less than without BHB (164%) and close to the values attained under

intact KG. Increase in BHB dosage to 2 mmol/kg in the same type of experiment inverted consecutive seizure aggravation to alleviation, and the second seizure integral attained $41.7 \pm 18.3\%$ of the first response (Fig. 4B; P6–8; $n=5$; $p=0.03$). These results indicate that protection of the immature neocortex by natural KG from the epileptogenic process involves ketone bodies and in particular BHB.

It has been suggested that depolarizing GABA contributes to enhanced excitability of the immature brain (Cepeda et al., 2007; Dzhala et al., 2005; Dzhala and Staley, 2003; Khazipov et al., 2004a; Nardou et al., 2009). The anti-convulsant efficacy of the drugs altering polarity of GABA responses, such as NKCC1-blocker bumetanide, and the drugs increasing GABA concentration or conductance, such as benzodiazepines or barbiturates, is disease and model specific (Dzhala et al., 2008; Glykys et al., 2009; Isaev et al., 2005, 2007; Kilb et al., 2007; Richter et al., 2010; Tyzio et al., 2009) (see also (Cohen et al., 2002; Huberfeld et al., 2007)). We therefore studied whether changes in GABA signaling are involved in the changes in brain excitability caused by KG blockade. In this aim we studied the effects of the NKCC1 blocker bumetanide and positive allosteric modulator of GABA receptors diazepam using paired flurothyl exposure protocol. In the conditions of intact KG, treatment of animals with diazepam (5 mg/kg; P6–8; $n=7$) 10 min before the second flurothyl exposure resulted in a reduction of the second seizure scalar integral to $35 \pm 6.6\%$ (Fig. 5A; $p=0.001$), that corresponds to a three-fold reduction comparing to anticipated paired-pulse facilitation to 114% (Figs. 3A and 6B). In the conditions of KG blocked by mercaptoacetate and insulin administered prior to the first and second flurothyl exposures, diazepam caused an alleviation of the consecutive seizure to $51.7 \pm 10.6\%$ (Fig. 5B, P6–8; $n=7$; $p=0.001$), corresponding to a similar three-fold reduction when compared to an anticipated aggravation of 164% (Fig. 3C and 6B). Thus, diazepam was equally efficient in suppressing epileptic activity in the conditions of intact and blocked KG suggesting that GABA exerts mainly an inhibitory role in flurothyl-induced seizure generation and that this inhibitory role is largely unchanged after KG blockade. In the next experiment, we studied whether the anticonvulsive role of GABA is modified by bumetanide, an agent which blocks depolarizing action of GABA on the immature cortical neurons *in vitro* (Tyzio et al., 2006; Yamada et al., 2004). Under conditions of blocked KG, bumetanide (5 $\mu\text{mol/kg}$) was administered 60 min prior to the second flurothyl exposure. Consecutive seizure aggravation in these conditions was of $146.8 \pm 14.7\%$ (Fig. 5C and 6B; $n=5$; P6–7; $p=0.008$), that was not significantly different from the 164% increase in the absence of bumetanide (Fig. 3C and 6B). Thus, in the flurothyl model, bumetanide does not exert anticonvulsive actions and GABA remains inhibitory even under conditions of blocked ketogenesis in contrast with previous data obtained *in vitro* (Rheims et al., 2009) and in keeping with the model-specificity in the anticonvulsive actions of GABA found *in vitro* (Dzhala et al., 2010; Kilb et al., 2007).

Discussion

The main finding of the present study is that natural ketogenesis occurring in the neonatal suckling rats controls brain

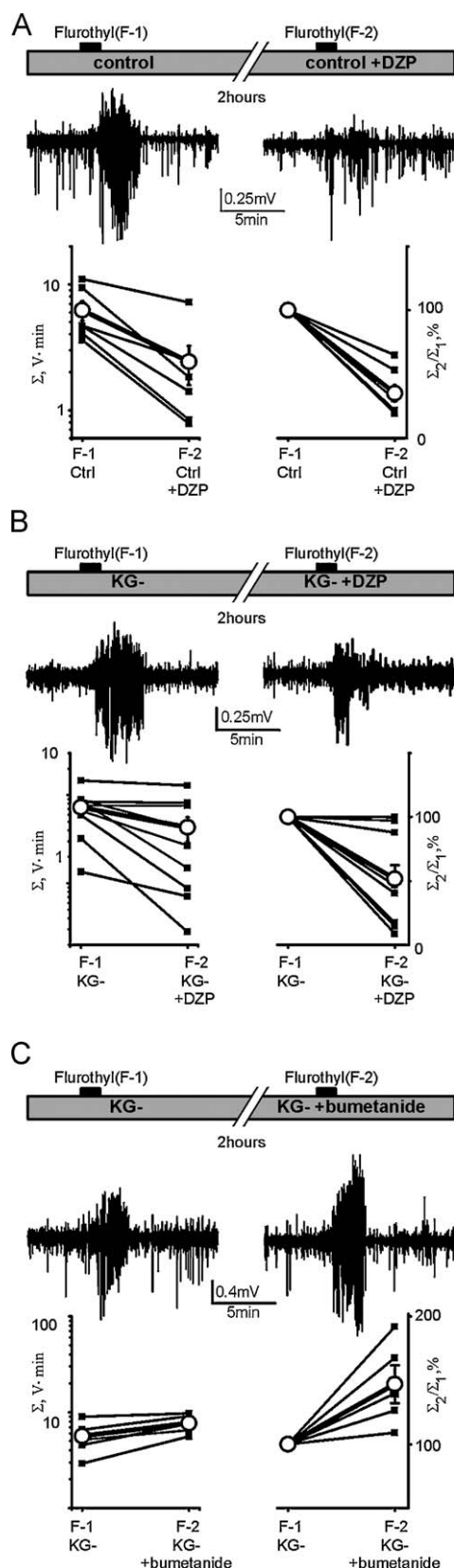


Figure 5 Effects of the GABA-acting drugs on consecutive seizures. (A and B) In the conditions of intact (A, P6–8, $n=7$) and blocked (B, P6–8, $n=7$) ketogenesis, positive allosteric modulator of the GABA(A) receptors diazepam (5 mg/kg) was

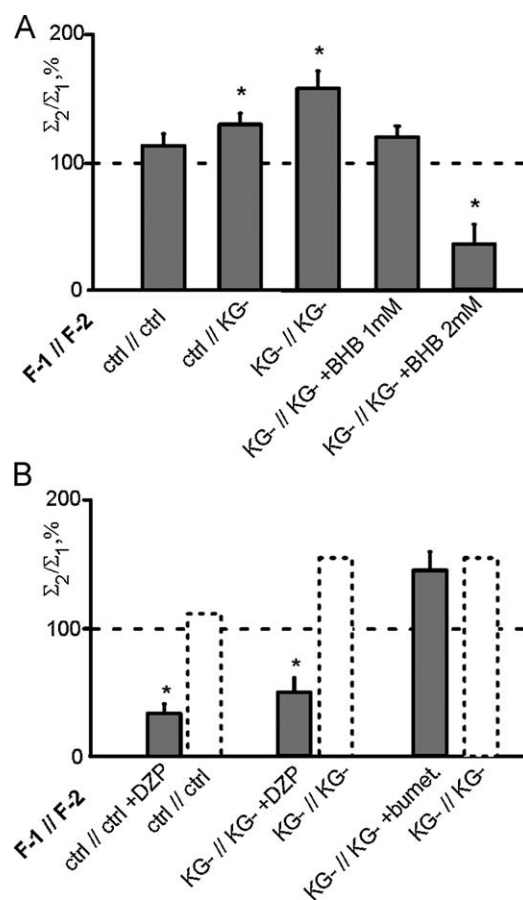


Figure 6 Summary plots of the repetitive flurothyl exposure experiments. (A) Ratio of scalar integrals of the first and second seizures ($\Sigma_2/\Sigma_1, \%$) in different ketogenic conditions. Abscissas code: first flurothyl condition (F-1)/second flurothyl condition (F-2). Note that blockade of ketogenesis (KG-) augments consecutive seizure aggravation and that exogenous BHB at physiological concentration (1 mmol/kg) reduces it back to control levels, and further augmentation of BHB levels to 2 mmol/kg reverses aggravation to alleviation. (A) Effects of the GABA-acting drugs (diazepam and bumetanide) on the repeated seizures ratio. Columns outlined with dashed lines correspond to the anticipated values obtained under similar conditions (from the plot A). Note that diazepam induces three fold consecutive seizure suppression both in the conditions of intact and blocked ketogenesis, and that bumetanide does not significantly modify consecutive seizures under conditions of blocked ketogenesis.

excitability and its blockade rapidly, within hours, facilitates seizure build up during repetitive seizures. We provide evidence that the protective effect of natural ketogenesis involves the anticonvulsive action of the ketone body BHB, and that seizure accentuation during two consecutive

administered 10 min before the second flurothyl exposure. Note that diazepam exerts powerful anticonvulsive effect in both conditions. (C) In the conditions of blocked ketogenesis, NKCC1 cotransporter blocker bumetanide (5 μ mol/kg, P6–7, $n=5$) administered 1 h before the second flurothyl exposure, did not affect consecutive seizure aggravation.

seizures under blocked KG does not involve modification of GABAergic inhibition.

The KD is an efficient treatment in children epilepsy and its withdrawal may rapidly provoke seizure (Huttenlocher, 1976; Rubenstein, 2008). Yet suckling mammals are under natural ketosis due to rich fat milk content in breast milk (Bougnères et al., 1986; Ferre et al., 1978, 1986; Nehlig, 1999). Ketone bodies are able to cross blood–brain barrier as demonstrated in P7 rats by direct measurements of beta-hydroxybutyrate concentration in different brain regions (Lust et al., 2003) and an expression of monocarboxylic acid transporters (Rafiki et al., 2003). The main goal of the present study was to determine whether and how natural KG in suckling animals affects brain excitability. Comparison of the first flurothyl-induced seizures did not reveal any significant difference between the scalar seizure integrals in control animals and in the rat pups with pharmacologically blocked KG. These results suggest that blockade of natural ketogenesis does not significantly affect a single (first) seizure. However, important seizure aggravation was observed with repetitive seizures in the animals with blocked KG, in which second seizure aggravation attained nearly 160%. The kindling phenomenon of seizure aggravation with repetitive seizures is a hallmark of epileptogenic process of “seizures begetting seizures” (Gowers, 1901; Khalilov et al., 2003, 2005). Our results suggest that natural KG serves to protect from it and that KG blockade “opens a gate” for the epileptogenic process. Further studies are required to determine whether blocked KG facilitates generation of self-sustained epileptiform activity following multiple recurrent seizures as described *in vitro* (Ben-Ari and Gho, 1988; Khalilov et al., 2003, 2005), and development of epilepsy.

What are the mechanisms underlying the protective action of natural KG? Because seizures are associated with a massive consumption of energy compensated by increased cerebral brain blood flow (Young et al., 1985), and ketone bodies provide an important metabolic source for the immature brain (Bougnères et al., 1986; Ferre et al., 1978, 1986; Nehlig, 1999), and because metabolic deficits may cause multiple deleterious effects contributing to epileptogenesis (Wasterlain et al., 1993), it can be suggested that blockade of natural KG causes metabolic deficits resulting in an increase in brain excitability (Dzhala et al., 1999, 2000). That restoration of BHB levels by exogenous administration of BHB under conditions of blocked KG prevents seizure build up is also compatible with the “metabolic” hypothesis. Recently, it has been suggested that BHB blocks depolarizing and excitatory action of GABA on the immature neurons *in vitro* (Rheims et al., 2009) (but also see (Kirmse et al., 2010; Ruusuvaari et al., 2010; Tyzio et al., 2011)) raising a hypothesis that protective effects of natural ketosis involve modulation of the polarity of GABAergic transmission. However, several lines of evidence indicate that this paradigm is unlikely. Firstly, we found that diazepam, a positive allosteric modulator of GABA channels, exerts equally efficient anticonvulsant actions both in control conditions with intact KG and after blockade of KG. Secondly, bumetanide, blocker of the chloride loader NKCC1 which is known to render GABA inhibitory in the immature neurons, did not significantly modify the consecutive seizure aggravation that could be expected from the data obtained *in vitro* (Rheims

et al., 2009). These two findings indicate that in the flurothyl model, GABA exerts anticonvulsive actions independently on the ketogenic state. This is line with a the hypothesis that diverse actions of the GABA-acting drugs in the immature brain are model and disease-specific (Baram and Snead, 1990; Dzhala et al., 2005, 2010; Isaev et al., 2005, 2007; Khalilov et al., 1999; Khazipov et al., 2004a; Kilb et al., 2007; Nardou et al., 2009; Richter et al., 2010; Tyzio et al., 2009). Because the efficacy of GABA acting drugs depends on the polarity of GABA signals, and because multiple recurrent seizures cause depolarizing shift in the GABA reversal potential in the intact hippocampus *in vitro* (Dzhala et al., 2010; Khalilov et al., 2003, 2005), in further research it would be of interest to determine whether similar changes in GABA signaling and associated change in the action of ketogenic diet and the GABA-acting drugs as a function of a number of recurrent seizures also occurs *in vivo*.

In conclusion, elimination of natural KG results in a rapid removal from seizure protection during repetitive seizures. Our study raises concern that the changes in diet, and pharmacological manipulations such as glucose infusion, and pathologies such as hyperinsulinism which break natural ketosis, may be a potential risk factor for epileptogenesis in nursing infants. In the present study, the hazardous consequences of KG break were observed in the healthy rat pups exposed to flurothyl. It is likely that the hazard from KG elimination would be accentuated in the infants with background pathology associated with recurrent seizures. Typically infants who have seizures do not breast feed and are given IV fluids with glucose. Our study suggests that this could be very detrimental, and indicates that even sick neonates should receive breast milk or receive hydroxybutyrate infusions to remain ketotic.

Acknowledgements

We thank Y. Ben-Ari, G.L. Holmes for the comments on the manuscript. Financial support from EU (EC contract number LSH-CT-2006-037315 (EPICURE) FP6 -Thematic priority LIFESCIHEALTH), INSERM, ANR, FRM, LFCE, Rothschild/Inserm “interface” programs is acknowledged.

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