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CRITICAL REVIEW AND INVITED COMMENTARY

Excitatory action of GABA on immature neurons is not due to absence of ketone bodies metabolites or other energy substrates

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Summary

Brain slices incubated with glucose have provided most of our knowledge on cellular, synaptic, and network driven mechanisms. It has been recently suggested that γ-aminobutyric acid (GABA) excites neonatal neurons in conventional glucose-perfused slices but not when ketone bodies metabolites, pyruvate, and/or lactate are added, suggesting that the excitatory actions of GABA are due to energy deprivation when glucose is the sole energy source. In this article, we review the vast number of studies that show that slices are not energy deprived in glucose-containing medium, and that addition of other energy substrates at physiologic concentrations does not alter the excitatory actions of GABA on neonatal neurons. In contrast, lactate, like other weak acids, can produce an intracellular acidification that will cause a reduction of intracellular chloride and a shift of GABA actions. The effects of high concentrations of lactate, and particularly of pyruvate (4–5 mM), as used are relevant primarily to pathologic conditions; these concentrations not being found in the brain in normal ''control'' conditions. Slices in glucose-containing medium may not be ideal, but additional energy substrates neither correspond to physiologic conditions nor alter GABA actions. In keeping with extensive observations in a wide range of animal species and brain structures, GABA depolarizes immature neurons and the reduction of the intracellular concentration of chloride ($[Cl^-]_i$) is a basic property of brain maturation that has been preserved throughout evolution. In addition, this developmental sequence has important clinical implications, notably concerning the higher incidence of seizures early in life and their long-lasting deleterious sequels. Immature neurons have difficulties exporting chloride that accumulates during seizures, leading to permanent increase of $[Cl^-]_i$ that converts the inhibitory actions of GABA to excitatory and hampers the efficacy of GABA-acting antiepileptic drugs.

KEY WORDS: GABA excitation, Intracellular chloride, Ketone bodies metabolites, Lactate, Pyruvate, Neonatal neurons.

Slice studies have provided most of our understanding of cellular and molecular mechanisms of synapse operation and network function, including short- and long-term synaptic plasticity, brain oscillations and their underlying mechanisms, heterogeneity of γ-aminobutyric acid (GABA)ergic interneurons and their roles in seizures, actions of voltage and transmitter-gated currents, roles of ion channels, synaptic processes, neurotransmitter receptors, and so on. These mechanisms have often been confirmed in vivo and have led to important therapeutic consequences. Given this history, slices have been considered as a good experimental ''compromise'' that enables investigators to study detailed mechanisms under excellent tissue conditions, while also avoiding conditions that are too far from the in vivo animal (such as neuronal cultures). Yet, slices obviously differ from the in vivo situation, as most of the extrinsic inputs and outputs are eliminated, as are the crucial organs that regulate the brain's function by hormones and modulators.

Numerous attempts have been made to alter artificial cerebrospinal fluid (ACSF) composition to reflect more faithfully the physiologic brain environment, including hormones and various modulators (Hajos & Mody, 2009; Hajos et al., 2009). In a recent series of studies (Rheims et al., 2009; Holmgren et al., 2010; Mukhtarov et al., 2011) and in a review (Zilberter et al., 2010), Zilberter et al. have postulated that slices relying on glucose (10 mM) as an energy substrate are energy deprived, and, therefore, other substrates such as lactate/pyruvate and 3-hydroxy-butyrate are mandatory to avoid energy deprivation. To illustrate the shortcomings of slice experiments, these authors challenged the well-known depolarizing action of GABA on immature rat neurons (Ben Ari et al., 2007) claiming that they are due

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to energy-deprived conditions of slices relying only on glucose as their energy substrate. Herein we review publications that show that ''sweet'' slices from 6–8 day old rats—perfused with the usual 10 mM glucose—are: (1) not energy-deprived; (2) have a ''proper'' energy state ([ATP]); (3) do not shift GABA actions when physiologic concentrations of other energy substrates are added; and (4) exhibit unintended features (and reflect pathologic conditions) when lactate/pyruvate at higher concentrations are added. We then review the wide range of observations that substantiate the GABA shift during development, briefly discuss the problems of in vivo determination of GABA actions, and consider the clinical implications of these experimental observations.

Energy Substrates and Energy State in Brains of 6- to 8-Day-old Rats

It is not clear exactly how Zilberter et al. (2010) define energy-starvation, since they have not measured metabolism. However, conventionally, energy starvation is interpreted as meaning a low level of [ATP] (and [CrP]) and/or [ATP]/[ADP] and [CrP]/[Cr] in the preparation. Moreover, Zilberter et al. are not explicit about whether their postulate is applicable to studies on tissue from all animal species and all ages, or only to their specific experimental material. It has been known for a long time (Lipton & Whittingham, 1982) that ATP content of slices from adult animals is low as compared with that in the whole brain. Reviewing the vast body of literature on the topic is beyond the scope of this article. We confined our considerations, rather, to the situation of brain slices from 6- to 10-day-old rats, showing that immature slices are neither energy-deprived nor altered in their ''normal'' functions by supplementation of other energy substrates at physiologic concentrations.

Energy substrates in blood and brain from 6- to 10-day-old rats

The serum concentration of lactate is about 1 mM, that of 3-hydroxy-butyrate (BHB) is 0.4–1.3 mM, and that of pyruvate is 0.14–0.16 mM (Page & Williamson, 1971; Schroeder et al., 1991; Nehlig & Pereira de Vasconcelos, 1993; Yager & Hartfield, 2002; Yager et al., 2002) and in the whole brain of 1.3–2.0 mM BHB, 1.3–1.7 mM lactate, and 0.08–0.09 mM pyruvate (Duffy et al., 1975; Vannucci et al., 1996; Yager & Hartfield, 2002; Yager et al., 2002). Direct measure of lactate in the umbilical and uterine vessels of Dorset ewes indicates that lactate accounts for only one-fourth of the energy supply, the remaining 75% being provided by glucose (pyruvate levels being low) (Burd et al., 1975). Mean arterial oxygen pressure in blood from adult animals is about 100 mm Hg, whereas brain oxygen tension is generally low and nonuniform (Erecinska & Silver, 2001; Erecinska et al., 2004). Brain levels of these agents in immature animals are not known but brain concentrations of fuels in immature animals are likely to depend on those in plasma, just as they are in adult animals (Silver & Erecinska, 1998). The impact of the in utero immature blood–brain barrier on these parameters is not clear but likely will more readily approximate that of the adult.

Conclusions

Concentrations of glucose and ketone bodies in brains of 6- to 10-day-old rats are lower than those in blood, whereas that of lactate is about the same (also see below). Interestingly, because the extracellular volume fraction (extracellular space) at 6–10 days old is considerable larger (0.25–0.40) than at 3 weeks of life (0.19–0.23) (Lehmenküler et al., 1993), the brain ''reservoir'' of substrates for the respiratory chain is larger in immature animals.

Energy substrates in slices from 6- to 10-day-old rats

In intact brain, fuels and oxygen are delivered through a network of blood vessels exquisitely sensitive to the needs of the organ. By contrast, in slices, this delivery is controlled by the speed of diffusion; that is, it is diffusion-limited. Uptake of glucose, lactate, and ketone metabolites by brain cells that occurs down the concentration gradient (facilitated diffusion) is not dependent on ATP. It was recognized in early studies that for this reason, maintenance of brain slices in a ''healthy'' condition requires a high oxygen tension and a higher than ''normal'' concentration of glucose (the predominant fuel for adult brain) in the artificial medium bathing the tissue. Hence, the ''standard'' superfusion solution contains 10 mM glucose and is equilibrated with 95% oxygen and 5% CO_2—despite the limitations of these conditions and the differences with the in vivo situation (PO_2 as low as 12 mm Hg) and the likelihood that the slice core is hyperoxic. Nevertheless, this large elevation in oxygen tension in slices does not fully compensate for the elimination of vascular circulation, at least in slices from adult rats; intrinsic optical signals of metabolism (NAD fluorescence) are different in vitro from those in vivo (Turner et al., 2007). In other words, the ACSF used for slice studies is a compromise between the need for sufficient energy supply and the lack of vascular system and its regulation by activity.

Whether or not lactate is a physiologic fuel for mature brain is still a matter of controversy (Dienel & Hertz, 2001; Pellerin & Magistretti, 2003; Hertz et al., 2007). Under physiologic conditions, neurons take up about 60% of the circulating glucose, despite the presence of astrocytic end-feet surrounding brain capillaries (Nehlig, 2004). In contrast, most adult animals utilize ketone bodies predominantly during starvation. The extent to which lactate and ketone bodies are used as fuels during development (Erecinska et al., 2004) depends on the animal species and age. Brains of rats at postnatal days 6–17 use

3-hydroxy-butyrate/acetoacetate as substrates in vivo (a metabolic preference that peaks after P10) (Page & Williamson, 1971; Nehlig & Pereira de Vasconcelos, 1993) but not lactate (Erecinska et al., 2004). This metabolic preference is due to the high content of fat in the rat's maternal milk, which puts the pups in a situation of nutritional ketosis (Dymsza et al., 1964); as during starvation, this situation acts as a signal indicating to the brain that glucose is less available. The brain rapidly adapts to this situation by using ketone bodies formed from free fatty acids by the liver to spare glucose for more specific brain needs like the pentose phosphate shunt, which is the only source of nicotinamide adenine dinucleotide phosphate (NADPH) in the brain and is necessary for brain lipid synthesis (Nehlig & Pereira de Vasconcelos, 1993).

Ketone bodies are not mandatory substrates for the growth and normal brain development of rat pups in vivo. Auestad et al. (1989, 1990) have artificially reared rat pups from 4–17 days on a rat milk in which medium chain triglycerides are isocalorically substituted by a glucose polymer, thereby reducing dramatically the levels of circulating ketone bodies. These authors reported that the rats had similar developmental parameters as seen in rat pups raised on maternal milk, including: (1) the concentrations of brain lipids, (2) the activities of ketone-body utilizing enzymes and pyruvate dehydrogenase, (3) and the gross organization and histologic arrangements of the brain, as well synaptic contacts and corticofugal and corticopetal fibers. Moreover, glucose remains a mandatory substrate in the state of nutritional starvation that characterizes the immature rat brain, and its specific regional utilization increase parallels the acquisition of the major physiologic functions (Nehlig, 2004).

Conclusions

The lack of a functional vascular network in in vitro systems requires increase in the levels of substrates for mitochondrial oxidative phosphorylation (oxygen and fuels) to above physiologic levels. High oxygen tension may increase generation of oxygen free radicals and cause oxidative damage in longer-lasting experiments. Lactate and ketones are weak acids that will transiently alter intracellular pH (see below). In addition, lactate alters the cytosolic redox state toward more reduced values in adult slices, and competes with glucose for NAD^+, thus inhibiting its oxidation (Gilbert et al., 2006).

ATP and effects of energy deprivation in slices from 6- to 10-day-old rats

ATP content of slices from adult animals is low as compared with that in the whole brain (Lipton & Whittingham, 1982). However, it is well established that immature animals, including rats, are far more resistant than adults to oxygen deprivation from both a functional (brain electrical activity) and metabolic view (potassium leakage, levels of high energy phosphate compounds) (Duffy et al., 1975; Hansen, 1977; Erecinska et al., 2005) due to their much slower rates of ATP utilization. The same is true for slices from immature rats (Kawai et al., 1989; Nabetani et al., 1995; Wada et al., 1997). In early studies, we tested directly this difference by recordings from neurons from adult and immature slices, placed in the same chamber to generate identical external conditions: The field excitatory postsynpatic potentials (EPSPs) recorded during oxygen (and/or glucose) deprivation were blocked in minutes in adults but persisted for long periods in the neonatal slice (Cherubini et al., 1989). The rate of utilization of high-energy phosphates in slices from a 7-day-old rat is 60% that of an adult, and this difference is reflected in a much lower rate of oxygen consumption in the former (almost threefold) (Kawai et al., 1989). ATP and CrP contents in 4- to 10-day-old rat brain slices are high and very similar to those in whole brain, whereas they are much lower in mature animals (Wada et al., 1997). Finally, the suggestion that an energy deficiency in immature slices accounts for the depolarizing GABA response cannot be readily reconciled with the observation that GABA produces isoelectric or hyperpolarizing currents in adult neurons (in glucose milieu see Glickfeld et al., 2009; Tyzio et al., 2008)—although adult slices are far more susceptible to energy insufficiency than immature slices. Along similar lines, GABA depolarizes newly born granule cells in the adult hippocampus in a glucose milieu (as well as olfactory neurons) while it inhibits adjacent ''adult'' neurons in the same preparation. This observation strongly suggests that the GABA developmental shift is an intrinsic rule and not determined by external conditions (Ge et al., 2006).

Conclusions

Concentrations of ATP and CrP in slices from 6- to 10-day-old rats are the same as those in whole immature and adult brain. High (5 mM) lactate and high (10 mM) BHB can maintain [ATP] but not normal brain activity (see above). In addition, if the hypothesis presented by Zilberter et al. is correct, adult slices (that are far more susceptible to energy deprivation) should be under severe metabolic stress and have depolarizing GABA and generate seizures and other pathologic activities; this is clearly not the case.

Conclusions on metabolism

Slices from brains of 6- to 10-day-old rats perfused with medium containing 10 mM glucose are not ''energy-starved,'' as demonstrated by their ''normal'' content of high-energy phosphate compounds and resistant synaptic responses. Because we are not aware of any evidence that a combination of respiratory substrates could increase ATP to a ''supranormal'' level (or that this increase would be beneficial), it is reasonable to conclude that energy starvation cannot be the reason for differences in experimental observations on GABA effects. Delivery of oxygen and fuels in

slices is diffusion-limited, thus requiring higher than normal levels in their external (ambient) concentrations. This condition may have unforeseen consequences, perhaps having more serious effects for nonphysiologic concentrations of weak acids than those of glucose and oxygen.

The Developmental GABA Actions Shift Is Not Conditioned by Additional Energy Metabolites

The key players in GABA actions and how they are measured: a brief outline

Neuronal chloride homeostasis is controlled by chloride cotransporters, exchangers, and channels (Plotkin et al., 1997; Delpire, 2000; Payne et al., 2003; Mercado et al., 2004). Particular attention has been devoted to the developmental changes in two cation-chloride cotransporters, NKCC1 that imports chloride and KCC2 that extrudes it—that appears to play a pivotal role in the developmental changes in intracellular concentrations of chloride ($[Cl^-]_i$). Agents that block NKCC1—like the diuretic and specific NKCC1 antagonist bumetanide—reduce $[Cl^-]_i$ and shift the polarity of GABA actions (Delpire, 2000; Dzhala et al., 2005; Tyzio et al., 2006; Nardou et al., 2011). Activation of $GABA_A$ receptors produces opening of the $GABA_A$-channel pore and thus allows Cl^- transfer according to electrochemical gradient (i.e., driving force, DF_{GABA}), which is the difference between the neuronal membrane potential (E_m) and the equilibrium potential of chloride (E_{GABA}). Early in development, GABA exerts depolarizing and excitatory actions (Cherubini et al., 1991; Ben-Ari et al., 1997; Ben Ari, 2002; Owens & Kriegstein, 2002; Ben Ari et al., 2007). These age-dependent properties of GABA signaling are determined by the progressive negative shift in reversal potential of GABA-mediated synaptic and extrasynaptic currents (E_{GABA}) that in turn reflects the gradual reduction of $[Cl^-]_i$. In other words, in immature neurons, activation of $GABA_A$ receptors produces outward flow of chloride (which corresponds to inward electrical current) and this result in depolarization of membrane potential and can excite the cell. In adult cells, GABA produces inward chloride flux (outward electrical current) and hyperpolarization of membrane, which decrease neuronal excitability and the generation of action potentials. The determination of resting membrane potential and DF_{GABA} with invasive recording techniques is hampered in immature neurons by their very high input resistance, necessitating the use of noninvasive cell-attached recordings of single ion channels in individual neurons (Tyzio et al., 2003, 2006). A very useful and reliable technique consists of measuring of E_m and DF_{GABA} from the same cell—with a dual recordings of single *N*-methyl-D-aspartate receptor (NMDA-R) channels to determine E_m and GABA channels to determine DF_{GABA}, thereby allowing precise calculation of E_{GABA} and study of developmental and pathologic changes of chloride homeostasis (Tyzio et al., 2003, 2006, 2008, 2009, 2011; Nardou et al., 2011). Figure 1 illustrates this technique with the estimation of E_{GABA} using double recordings of DF_{GABA} and E_m from the same neocortical cell. The intersection of these current/voltage relationships (I/V curves) with baseline provides a reliable estimate of E_m—for the NMDA-R single channel recordings—and DF_{GABA}—for the GABA single channel recordings—and the difference between them provides an adequate estimation of E_{GABA}.

Physiologic concentrations of energy substrates do not alter GABA depolarizing actions on neonatal neurons

An abundant literature cannot be reconciled with the suggested link between GABA actions on immature neurons and ketone body metabolites. Therefore, the time course of the GABA shift (from depolarization to hyperpolarization)—roughly completed by the end of the first postnatal week for CA1 hippocampal neurons (Ben Ari et al., 2007)—occurs much earlier than the maturation of ketone body metabolites, the transporters that import them, and BHB dehydrogenase that metabolizes them (Nehlig & Pereira de Vasconcelos, 1993). The brain preserves physiologic levels of glucose even under severe metabolic stress (fasting for several weeks), and immature (like adult) slices cannot sustain physiologic activity in the absence of glucose (see above). GABA depolarizes similarly immature chick spinal cord neurons, as is also the case in *Xenopus* oocytes, Zebrafish, or worms where maternal milk is not a significant factor but that express a developmental alteration of the NKCC1/KCC2 ratio and a corresponding shift in the GABA depolarizing/hyperpolarizing action (Ben Ari et al., 2007) (and see below). The main well-recognized action of ketone body diets—its antiepileptic actions on specific forms of early seizures—are not thought to be mediated by GABA signals, since the ketogenic diet blocks seizures generated by GABA receptor antagonists (Appleton & De Vivo, 1973; Appleton & DeVivo, 1974; Bough & Eagles, 1999; Bough et al., 2000; Thio et al., 2000; Sullivan et al., 2003; Hartman et al., 2007; Yellen, 2008; Maalouf et al., 2009). Other mechanisms underlying the seizure-inhibitory action of ketone body metabolites have been suggested, including a direct reduction of glutamate vesicular release that will efficiently reduce ongoing activity (Juge et al., 2010) and an action of KATP channels (Yellen, 2008). It may be worth also remembering the inhibitory actions of GABA in adult glucose-incubated slices, despite their higher vulnerability to energy deprivation than immature neurons. The observations that GABA exerts cell- and sex-specific actions on neurons belonging to the same neuronal population can be explained only with considerable difficulty by the metabolic deprivation hypothesis (Ben Ari et al., 2007 and see below).

Despite all of this evidence, we have recently retested the actions of these energy metabolites, using identical

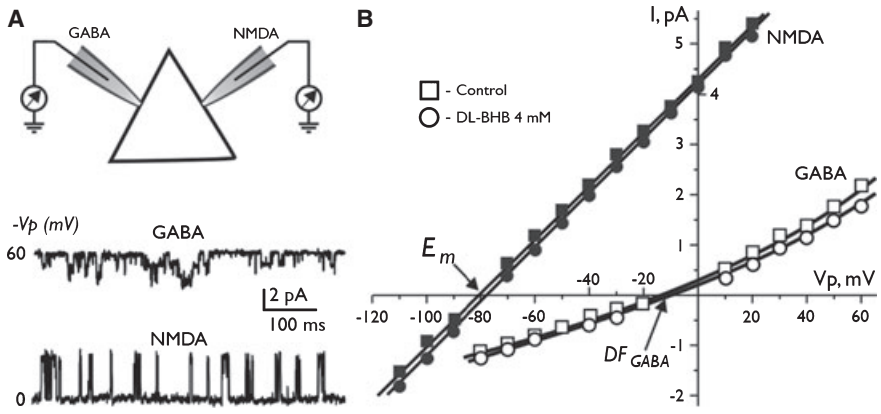

Figure 1.
Estimation of E_{GABA} by double recordings of DF_{GABA} and E_m from the same neocortical cell. (**A**) Scheme of consecutive cell-attached recordings of single NMDA-R and $GABA_A$–R channels. First we recorded from the same neuron in control conditions NMDA-R channels (for E_m) then $GABA_A$-R channels (for DF_{GABA}). These two measurements allow us to determine E_{GABA} for this neuron. Then, we applied DL-BHB for 40 min and repeated the recordings with the same sequence (NMDA-R and then $GABA_A$-R channels). Therefore, every cell studied was patched four times. Representative traces of recordings of the single channels openings are shown below. (**B**) Representative plot of I-V relationships of single $GABA_A$-R and NMDA-R channels used for estimation of E_{GABA} in neocortical pyramidal cell (E_{GABA} = DF_{GABA} + E_m). Each point is mean amplitude of about 30 openings at a given pipette potential (Vp). The reversal potential that corresponds to DF_{GABA} was estimated by the exponential growth fit of the I–V curve. The current-voltage relationships of NMDA-R channels were best fitted with linear function (see also Tyzio et al., 2003, 2008). Note that application of DL-BHB does not change significantly DF_{GABA} and E_m. (Reproduced with permission from Tyzio et al., 2011).
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conditions as Zilberter et al. (Rheims et al., 2009; Holmgren et al., 2010) but with a wide range of recording and imaging techniques that extend from noninvasive single channel recording to noninvasive multibeam two-photon calcium imaging techniques to visualize the activity of hundreds of neurons (Crepel et al., 2007; Bonifazi et al., 2009). We found (Tyzio et al., 2011) that additional energy metabolites at physiologic concentrations had no effects on GABA signals (Figs 1 and 2). Therefore, BHB (4 mM), did not alter: (1) resting membrane potential, driving force for GABA and reversal potential of GABA in somatic recordings of single $GABA_A$-R and NMDA-R channels (Fig. 1); (2) E_m, synaptic GABAergic potentials, and the responses evoked by GABA application in gramicidin perforated patch recordings; (3) spikes generated by GABA in cell-attached recordings; and (4) frequency of giant depolarizing potentials (GDPs) (Ben-Ari et al., 1989)—the first synapse driven network pattern that drives immature hippocampal networks to generate synchronized activities or the earlier intrinsic synapse independent synchronous calcium plateaux generated by synchronous plateau assemblies (SPAs) as determined with calcium imaging techniques (Fig. 2) and electrophysiologic recordings (Tyzio et al., 2011). Figure 2 shows the lack of effects of BHB on the patterns generated by hundreds of neurons. Similar observations were made recently in the upper neocortical layers using perforated patch recordings and imaging techniques (Kirmse et al., 2010). Figure 2 also shows the efficient actions of bumetanide that blocks GDPs and reduces ongoing network activity; this serves as a useful control to evaluate the roles of depolarizing GABA actions on network activity.

Interestingly, earlier studies had shown the presence of a contaminant dibenzylamine in some BHB sources that act on various channels that appears to mediate the actions thought to be mediated by BHB (Doepner et al., 1997, 2001; Rho et al., 2002; Donevan et al., 2003) raising strong concern as to the reliability of conclusions derived from BHB experiments. We discovered that one of the BHB sources used in the laboratory (Acros Organics) indeed contained this contaminant and showed that this efficiently altered DF_{GABA} and corresponding brain patterns as viewed with our imaging techniques. Therefore, care must be exerted when using BHB and tests made to ensure that the contaminant is not present to avoid misleading interpretations of the actions of BHB. Therefore, ketone body metabolites do not alter GABA signals in immature neurons, whereas blocking NKCC1 exerts a dramatic action on both GABA actions and network activity.

The NKCC1/KCC2 developmental sequence is instrumental to the GABA shift

An important argument developed by Zilberter et al. (2010) (also see Rheims et al., 2009; Mukhtarov et al., 2011) is that the levels of $[Cl^-]_i$ are determined in immature neurons by NKCC1 and the HCO_3^- system, and not (as usually thought) by the NKCC1/KCC2 developmental

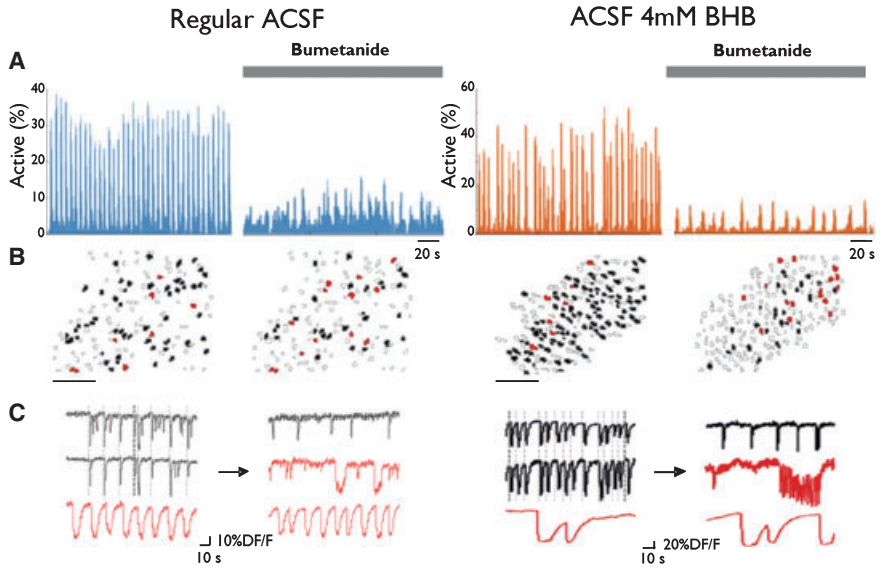

Figure 2.
DL-BHB does not alter spontaneous neuronal activity patterns in neocortical slices. (**A**) Histograms indicating the fraction of active cells as a function of time in calcium movies in regular ACSF and in the presence of 4 mM DL-BHB (Sigma-Aldrich, Lyon, France). Each peak of synchronous neuronal activity in the histograms corresponds to a GDP. GDPs were strongly reduced in the presence of the NKCC1 antagonist bumetanide (10 μM) in neocortical slices 27 from P7 rats. (**B**) Automatically detected contours of the imaged cells: Open contours indicate silent cells, black-filled contours indicate cells involved in GDPs, and red-filled contours are synchronous calcium plateaux in cell assemblies (SPA) cells. Note that the number of SPA-cells relative to the number of active cells increased in the presence of 10 μM bumetanide in the neocortex and hippocampus (scale bar: 100 μm). (**C**) Calcium fluorescence traces of representative cells implicated in GDPs (black) and in SPAs (red). Note that some GDP cells display an SPA pattern of activity after adding bumetanide (middle traces). Reproduced with permission (Tyzio et al., 2011).

sequence. However, HCO_3^- has no major influence on E_{GABA} in neonatal neurons because the internal $[Cl^-]_i$ concentration is high (Farrant & Kaila, 2007), and becomes fully operational only after the NKCC1/KCC2 developmental shift has taken place (Ben Ari et al., 2007). In contrast, the NKCC1/KCC2 developmental sequence has been confirmed in all animal species investigated, from invertebrates to humans (Dzhala et al., 2005; Akerman & Cline, 2006; Liu et al., 2006; Howard et al., 2007; Kahle & Staley, 2008; Glykys et al., 2009). NKCC1 knockout mice do not have depolarizing GABA actions or GDPs (Pfeffer et al., 2009; Sipila et al., 2009), and the specific NKCC1 antagonist, bumetanide—at least with low concentrations—shifts GABA actions both in vivo and in vitro (Wang & Kriegstein, 2008, 2010). Cells that do not express KCC2 in development or adulthood have depolarizing GABA (Price et al., 2005; Gilbert et al., 2007; Pozas et al., 2008), and genetically determined over- or underexpression of KCC2 alters GABA polarity, GABA synapse formation, and neuronal development in vivo (Chudotvorova et al., 2005; Lee et al., 2005; Ben Ari et al., 2007; Cancedda et al., 2007; Wang & Kriegstein, 2008). Enriched environment shifts GABA actions earlier (He et al., 2010), and the delayed expression of KCC2 and E_{GABA} is cell- and sex-specific (Kandler & Friauf, 1995; Kandler et al., 2002; Balakrishnan et al., 2003; Gulacsi et al., 2003; Lee et al., 2005; Lohrke et al., 2005; Banke & McBain, 2006; Blaesse et al., 2006; Kim & Trussell, 2009; Belenky et al., 2010). Although other mechanisms have been suggested to explain the shift in GABA polarity (Howard et al., 2007), its determination by the NKCC1/KCC2 developmental shift has been confirmed by an extensive and abundant literature.

Alterations of GABA actions by high lactate/pyruvate are due to intracellular acidosis not to metabolic improvement

Weak acids produce an intracellular acidification that will directly cause a reduction of $[Cl^-]_i$. This mechanism has been excluded by Zilberter et al. (2010) and Mukhtarov et al. (2011) since (1) field recorded GDPs were preserved in an acidic bicarbonate-free HEPES solution, and (2) the acidification observed with lactate was too small to produce significant effects. However, the GDPs reported in an acidic milieu have much higher amplitude than "controls," and concomitant field and perforated patch recordings are required to ensure that these are indeed bone fide GDPs and

not interictal events that in keeping with the larger contribution of glutamate receptor mediated currents in their generation also have a more depolarized reversal potential than GDPs (Nardou et al., 2009).

Recently, Kaila and coworkers (2011) have monitored the mitochondrial membrane potential and intracellular pH in CA3 neurons in neonatal rat hippocampal slices and provided direct evidence that intracellular acidification—not metabolic demands—underlies the effects of these weak acids (Fig. 3). Therefore, reducing glucose caused a gradual and reversible depolarization of the mitochondrial membrane potential, regardless of whether 5 mM L-lactate was present or not (Ruusuvuori et al., 2010). In addition, L-lactate, a well-known substrate of oxidative energy metabolism (Erecinska et al., 2004; Raichle & Mintun, 2006), produced a transient fall in intracellular pHi that was closely paralleled by a suppression of GDPs. The subsequent recovery of pHi in the continuous presence of L-lactate was associated with a recovery of GDPs that were blocked by bumetanide. Most importantly, nonmetabolized weak acids, including propionate and D-lactate, produced similar effects on pHi and GDPs as L-lactate (which is metabolized), clearly indicating that lactate's effects are attributable to the transient fall in pHi and not to metabolic improvement (Fig. 3). In keeping with this finding, the intracellular acidification produced by increasing CO_2 (to 8%) led to a transient and temporally coincident reduction of GDPs (Ruusuvuori et al., 2010), reflecting both the impact of HCO_3^- and intracellular acidification on GABA actions. Extensive investigations suggest that intracellular acidification impacts on neuronal excitability (Roos & Boron, 1981; Chesler, 2003) From these experiments, it seems clear that the effects of lactate and pyruvate are due to acidification not to metabolic improvement.

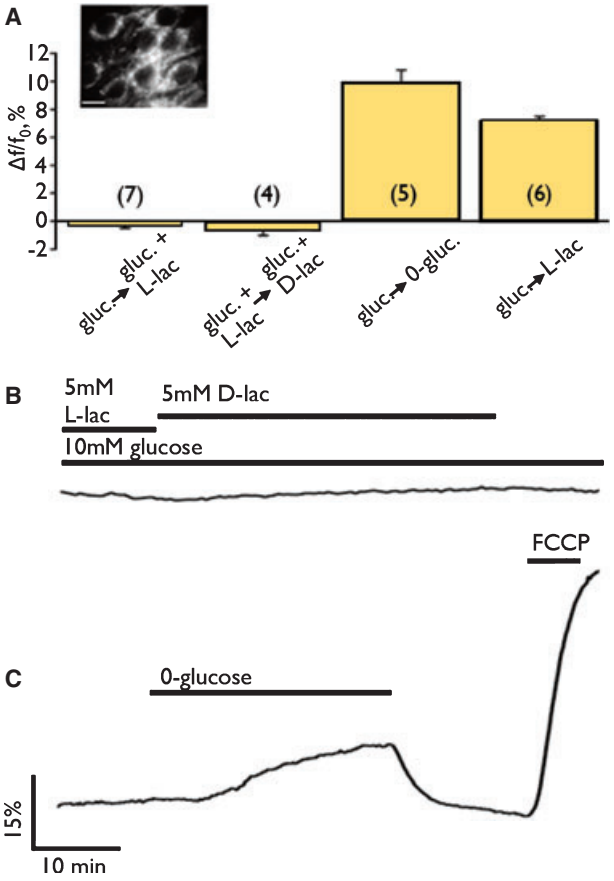

Figure 3.
Depolarizing GABA actions are not attributable to deficient mitochondrial energy metabolism. (**A**) Mitochondrial membrane potential is not affected by 5 mM L-lactate (L-lac) or when L-lactate is replaced with D-lactate (D-lac). The bar diagrams represent the normalized Rh123 fluorescence of CA3 pyramidal neurons [± standard error of the mean (SEM), number of slices shown in brackets]. Inset, CA3 pyramidal neurons in a slice preparation loaded with Rh123. Scale bar, 10 mM. (**B**) A specimen recording showing the lack of effect when 5 mM L-lactate in standard solution is replaced with 5 mM D-lactate. (**C**) Glucose (gluc) depletion causes a gradual and reversible depolarization of the mitochondrial membrane potential. When carbonyl cyanide4-(trifluoromethoxy) phenylhydrazone (4 μM) is applied to uncouple mitochondrial membrane potential, a profound increase in fluorescence intensity takes place, reflecting a depolarization of mitochondria. Reproduced with permission from Ruusuvuori et al., 2004, 2010.

High concentrations of lactate and pyruvate are signatures of severe pathologic conditions

In their review, Zilberter et al. (2010) indicate that the brain levels of lactate and pyruvate are higher than plasma levels in humans under physiologic conditions, thereby justifying the use of ~5 mM lactate/pyruvate. However, adult rodent brain lactate/pyruvate levels in the studies cited in their review are much lower (between 0.9 and 2.7 mM for lactate and about 120 μM for pyruvate), with the classical 10-fold lactate/pyruvate ratio (Table 1 of Zilberter et al., 2010). Extensive investigations suggest that brain lactate is not higher than serum levels, at least in physiologic conditions (Table 1 and see below). Interpretation of the high values of lactate observed with imaging techniques and/or push pull cannula in humans on which Zilberter et al. (2010) rely are handicapped by the use of anesthesia that alters metabolism (Lowry & Fillenz, 2001) and the local damage produced by the cannula (Gallagher et al., 2009). In addition, these values are made in patients with epilepsies, head trauma, and or tumors and are, therefore, not relevant to physiologic conditions. Furthermore, high millimolar levels of pyruvate have not been observed even in these conditions.

Relying on their observations in slices and the suggested importance of additional energy substrates, Zilberter et al. (2010) suggest that high lactate and pyruvate will be useful to treat "Alzheimer disease, Leigh syndrome, epilepsy, dementia, multiple sclerosis, neuropathies or ataxia because

Table 1. Brain and serum levels of glucose, lactate, and pyruvate in clinical and experimental conditions in controls and after insults[a]

	Glucose		Lactate		Pyruvate			
	Plasma	Brain	Plasma	Brain	Plasma	Brain	Comments	References
Rat (6–10 days)								
Control	4.6–6.4	1.3–2.0	0.8–1.2	1.3–2.0	0.14–0.16	0.08–0.09		Page & Williamson, 1971; Nehlig & Pereira de Vasconcelos, 1993; Schroeder et al., 1991; Duffy et al., 1975; Yager & Hartfield, 2002; Yager et al., 2002; Vannucci et al., 1996
Hypoxia	5.8	0.45	11.3	10.5			Hypoxia in 10-day-old rats depletes brain glucose and increases lactate plasma and brain levels by 9–14 and 5- to 8-fold, respectively	Yager et al., 2002
Seizures	4.0	0.9	4.4	4.0			Seizures in 10-day-old rats slightly decrease brain glucose and increase lactate plasma and brain levels by 4–5 and 2–3, respectively	Yager et al., 2002
Rat (adult)								
Control	6.2–8.1	0.6–2.8	1.1–1.3	1.2–1.6	0.14–0.16	0.09–0.12		Siesjö, 1978; Passoneau et al., 1980; Rex et al., 2009
Hypoxia			6.3	3.5			After 60 min hypoxia, plasma levels of lactate increase about 5-fold and brain levels by 2.3-fold	Harada et al., 1992
Seizures				3.0-5.0		0.12-0.18	Brain lactate is increased by 2.5–3.0-fold during seizures	Duffy & Plum, 1981
Human								
Control	5.5	0.82–1.7	0.8	1.4–2.9		0.17		Roslin et al., 2003; Cavus et al., 2005; Occhipinti et al., 2011
Epileptic								
Cortex		1.4		5.2			Brain lactate is increased by 2–5-fold in brain tissue of epileptic humans	Cavus et al., 2005
Hippocampus		2.9		6.8				
Gliomas		1.4–2.2		7.9–8.8		0.13–0.14	Brain lactate is increased suggesting that gliomas are highly metabolically active	Roslin et al., 2003; Marcus et al., 2010

[a]Note that pyruvate levels in the brain or the serum are never higher than circa 200 nM. Note also that lactate is increased both in the brain and the serum after seizures and/or hypoxia.

of their suggested importance in neuronal metabolism.'' However, a very abundant literature (9,129 references in PubMed as of early March 2011—under the key words ''lactate'' and ''neurological disorders'') indicates what is well known to clinicians—that high brain lactate/pyruvate are signatures of neuronal injury. These high levels are observed in patients with epilepsies, traumatic brain injury, cancer melanomas, Leigh syndrome, acute brain injury, and so on (Detre et al., 1991; Cavus et al., 2005; Lee et al., 2009; Feuerstein et al., 2010). They are also accurate biomarkers for the outcome of neonatal encephalopathies (Gibson et al., 1981; Pavlakis et al., 1998; Sijens et al.,

2001; Dotti et al., 2004; Ross et al., 2010; Thayyil et al., 2010). Under experimental conditions, high pyruvate levels lead to seizures (Gonzalez-Falcon et al., 2003; Gonzalez et al., 2005), and injections of lactate in humans and rodents generate panic attacks by an oxidation of brain redox status and increased hippocampal firing (Bergold et al., 2009). Admittedly there are some experimental studies suggesting neuroprotective effects of lactate (quoted by Zilberter et al., 2010), but these effects appear to be mediated by antiinflammatory actions, not by metabolism amelioration (Suh et al., 2005; Cai et al., 2009). Genetic mutations of pyruvate dehydrogenase phosphate increase brain levels of pyruvate, decrease lactate, and produce a lethal infantile phenotype (Cameron et al., 2009). Therefore, it is not likely that injections of lactate/pyruvate will provide novel therapeutic strategies, and care must be exerted to suggest therapeutic implications relying on indirect experiments.

In vivo recordings are handicapped with severe limitations

In vivo experiments have provided evidence in favor of both depolarizing and hyperpolarizing actions of GABA (Fellippa-Marques et al., 2000; Ben Ari et al., 2007). However, although GABA inhibits neurons in neonatal rabbit hippocampal neurons, the use of sharp electrodes (with nearly 500 MOhm shunting conductance) introduces large errors in E_m in high membrane resistance immature neurons (Tyzio et al., 2003) that can ''translate'' into inhibitory GABA. Because the large shunting conductance also shifts the action potential threshold in a depolarizing direction, the low Cl^- conductance and the absence of Cl^- extrusion will augment the impact of Cl^- ions leaking from sharp electrodes. DF_{GABA} estimated using cell-attached recordings of GABA channels is depolarizing in hippocampal neurons in the in vivo superfused hippocampus preparation but urethane anesthesia affects intracellular homeostasis and the superfused conditions differ from in vivo conditions (Khazipov & Holmes, 2003; Tyzio et al., 2008). Preliminary cell-attached recordings of single GABA channels recordings from neocortical neurons under ketamine-xylazine anesthesia (S. Rheims, Ph.D. examination, Université de la Méditerranée) had a poor signal/noise ratio excluding any reliable conclusions (R. Khazipov, N. Burnashev, R. Tyzio, unpublished observations). The fundamental issue is the crucial need to record GABA channel currents in both directions. Although it is not difficult to record the currents carried by influx of chloride (with 130 mM chloride outside), the currents carried by efflux of chloride (a 10-fold lower intracellular chloride concentration) are of very small amplitude (fractions of picoampere) and the Godman-Hodgkin-Katz model predicts that conventional cell-attached recordings cannot resolve currents generated by very low intracellular chloride concentrations. Therefore, there is at present no reliable indication as to the actions of GABA in vivo.

We also doubt whether in vivo recordings will solve the problem and provide reliable compelling observations as they have severe intrinsic limitations. Blind cell-attached in vivo recordings are restricted to the somata of unidentified (probably large) neurons in which the GABA shift may have taken place and excluding dendritic recordings (not feasible with 6 MOhm pipettes) where GABAergic synapses are established first (Tyzio et al., 1999; Khazipov et al., 2001). They also cannot take into account the intraneuronal chloride gradients, a potential problem illustrated by the contradictory observations across various researchers on the actions of GABA on axons and somata, and the age-dependent differences in KCC2 distribution and GABA polarity (Ben Ari et al., 2007; Khirug et al., 2008; Glickfeld et al., 2009). This issue is particularly important in human and nonhuman primates where cortical neurons are generated along extended periods leading to an extreme heterogeneity of GABA shifts and demanding necessarily to identify the birth date of identified neurons in keeping with the recently reported differences between neurons born at different ages (Deguchi et al., 2011). Future advances will come from genetic cell and fate mapping that may facilitate the identification and visualization in vivo of the actions of GABA on specific cell types and possibly even cell compartments.

A central issue: the dynamic chloride regulation (DCR)

To some extent the values of $[Cl^-]_i$ at a given moment conveys only a partial information as this value is heavily altered by ongoing activity and its capacity to recuperate after a burst of spikes or a synchronized synapse driven network activity. Extensive evidence suggests that ongoing activity heavily impact on the polarity of GABA actions (Khalilov et al., 1999, 2003; Woodin et al., 2003; Khalilov et al., 2005; Fiumelli & Woodin, 2007; Balena & Woodin, 2008; Nardou et al., 2009). We have developed an approach that provides an approximation to the DCR (Nardou et al., 2011; see Fig. 4). We perform perforated patch-clamp recordings and apply GABA pulses via a nearby GABA containing electrode. The voltage is clamped at the equilibrium potential of GABA (E_{GABA})—and hence GABA pulses generate no current (Fig. 4). Then depolarizing voltage step is applied leading to influx of chloride and at the end of the pulse; the same GABA application now generates a large inward current attesting to an inward shift of E_{GABA}. We then determine the kinetic of recuperation, that is, the return to E_{GABA} that preceded the voltage step. This duration is a good indication of the activity of the chloride exporter KCC2 as it is dramatically enhanced when relatively selective KCC2 antagonists—but not NKCC1 antagonists—are applied (Fig. 4). Using this paradigm, we observed first that immature neurons have a much longer recuperation time than adults (not shown), providing direct evidence on the developmental sequence of the functionality of KCC2. We also observed that epileptic neurons have a

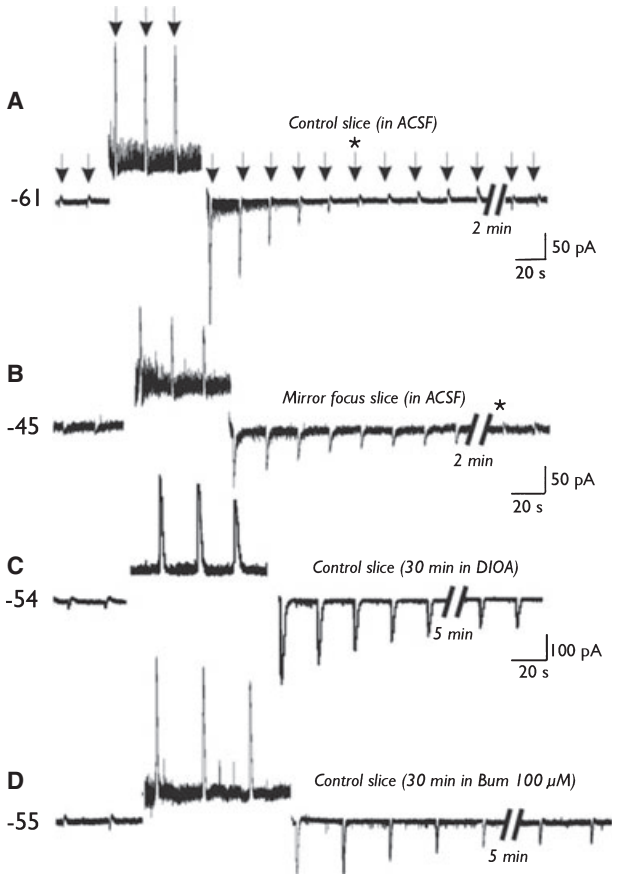

Figure 4.
Dynamic removal of chloride is severely hampered in mirror focus (MF) epileptic neurons. GABA was focally applied (arrows) to evoke a response in perforated patch-recorded neurons (in the presence of CNQX and APV) at a holding potential corresponding to E_{GABA}. A large depolarizing pulse from E_{GABA} to 0 mV (1 min) was applied and the delay to recovery of E_{GABA} was determined. (**A**) In a control neuron, after depolarizing pulse, GABA generated inward currents that progressively decayed to reach E_{GABA} after circa 1 min (*) (68.4 ± 7.9 s, n = 18). (**B**) In MF epileptic neurons, the duration was increased fivefold to circa 5 min (*) (306 ± 33.5 s, n = 5, $p < 0.001$). (**C**) In a similar protocol, the selective blockade of KCC2 with 10 μM R-(+)-[(2-*n*-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid (DIOA). (**D**) or 100 μM bumetanide (Bum) dramatically augmented this delay, E_{GABA} failed to recuperate even after 10–15 min (n = 7 and n = 6, respectively).

much longer time to recuperation (Nardou et al., 2011). Therefore, immature neurons are endowed with a reduced capacity to export chloride once this has been imported, and this is partly due to a less operative KCC2 (see below). The parallelism between immature neurons and epileptic ones is in keeping with the higher incidence of seizures in the developing brain and stresses the importance of the dynamic regulation of chloride after enhanced activity episodes.

Clinical implications

Epilepsies have a higher incidence early in life, and seizures can produce life-long severe sequela. Immature networks tend to generate seizure-like activities based on various intrinsic features, most notably their tendency to oscillate, the high input resistance of the connected neurons, and the difficulty of immature neurons to regulate the shifts of chloride that occur during synchronized activities. Following recurrent seizures, chloride accumulates and GABA becomes strongly excitatory (Khalilov et al., 2003; Khazipov et al., 2004; Dzhala et al., 2005; Khalilov et al., 2005; Nardou et al., 2011). This accumulation, in turn, both facilitates the generation of further seizures and reduces (and even reverses) the beneficial actions of GABA-acting antiepileptic agents such as phenobarbital or benzodiazepines (Dzhala et al., 2010; Nardou et al., 2011). Although, this condition is mediated by the continuous influx of chloride via NKCC1 (Dzhala et al., 2010), this chloride cotransporter is neither necessary nor sufficient, as these shifts of GABA polarity also occur in NKCC1 Kos (Nardou et al., 2011). The shift is primarily due to a downregulation and internalization of the chloride exporter KCC2 revealed with physiologic and anatomic techniques (Nardou et al., 2011). This is also in keeping with studies showing that this cotransporter is highly sensitive to tyrosine phosphorylation and seizures that control its turnover (Lee et al., 2010). The diuretic NKCC1 antagonist bumetanide blocks seizures with some but all models (Kilb et al., 2007) and ameliorates the actions of phenobarbital (Dzhala et al., 2005, 2010). Using our triple chamber preparation that is highly suited for that purpose as convulsive and anticonvulsive agents are applied on different networks(Khazipov et al., 1999), we showed more recently that bumetanide ameliorates the actions of phenobarbital, particularly when applied after a few seizures (Nardou et al., 2011). After many recurrent seizures, GABA strongly excites neurons, phenobarbital aggravates this action, and bumetanide fails to improve the situation significantly (ibid). This suggests that the history of seizures determines the efficacy of actions of GABA-acting antiepileptics and that the use of a diuretic is a promising strategy when combined with phenobarbital. An early use of the diuretic is also important in preventing as much as possible the internalization of KCC2 and thereby preserving the capacity of neurons to regulate their chloride levels. These observations have led to clinical trials on the use of the diuretic agent bumetanide, to produce a more favorable environment for the action of phenobarbital. Therefore, a large clinical trial on the use of bumetanide to treat 2-day-old babies with encephalopathy and phenobarbital-resistant seizures is now being performed (NEMO, FP7-EU clinical trial: http://www.nemo-europe.com/). Relying on the paradoxical actions of benzodiazepines in autistic patients that may suggest elevated intracellular chloride levels, Lemonnier & Ben-Ari (2010) recently tested the effects of bumetanide in five children (3–11 years old, suffering from autistic

syndrome disorders (ASDs) and found highly beneficial effects after a 3-month treatment with no side effects. A large double-blind test is now being pursued. Clearly, the alterations of intracellular Cl^- that are observed after a wide range of insults and disorders are a very promising target for novel therapeutic avenues (Lu et al., 2007; Boulenguez et al., 2010; Simard et al., 2010).

Therefore, immature neurons need to maintain a high intracellular chloride concentration (and excitatory GABA action) to enable a wide range of trophic actions that GABA exerts at the earliest developmental stages. This, however, also involves a high degree of instability in the neuron's ability to cope efficiently with chloride fluxes, potentially leading to pathologic clinical consequences. The developing brain is not a small adult brain but one with unique developmental sequences, molecular and cellular features, and a system that faces the problem of synchronizing highly heterogeneous neuronal populations to modulate—in an activity-dependent manner—the construction of functional ensembles.

General Conclusions

There is no doubt that slices are only pale reflectors of the in situ situation, and investigators continue to make suggestions to improve this compromised situation. One of the most limiting factors in establishing in situ–like conditions in slices is the lack of metabolic regulations and feedback loops that are instrumental in our reactions to the environment. For example, gluconeogenesis is heavily controlled by several organs, and increasing ketone bodies or sugar will alter general metabolic supply by directly altering glucose availability; and these complex loops cannot be reproduced in slices. However, slices from brains of 6- to 10-day-old rats perfused with medium containing 10 mM glucose are clearly not ''energy-starved'' and remain the most appropriate preparation to determine cellular and molecular mechanisms of brain operation.

When discovered more than two decades ago, the GABA developmental shift was considered a curious observation without fundamental implications. Studies performed in the last two decades have shown that this ''curiosity'' is a fundamental property of the developing brain. First, the GABA shift is but one of the many developmental sequences that enables immature neurons to act very differently from adult neurons. Almost every voltage- and transmitter-gated current has been shown to differ in young and adult neurons, in terms of subunit composition and operation. This developmental shift acts to adapt brain activity to the different functions of immature and adult networks. Immature neurons must have long-lasting ''sloppy'' currents to enable neurons to fire and wire together despite their large fate and birth heterogeneity. For example, in primates, cortical neurons divide over a period of up to 100 days, so some cells may have—at a given time point—no or few synapses, whereas adjacent cells will have thousands of functional synapses. Secondly, the actions of depolarizing GABA are made possible by the activation by GABA of other voltage-gated currents (notably the persistent sodium current—see Valeeva et al., 2010), and of NMDA currents (Leinekugel et al., 1997; Medina et al., 1999) facilitated by the presence of immature forms of NMDA-R channels endowed with less voltage dependence and longer kinetics (Pollard et al., 1993; Monyer et al., 1994). This interdependence underlies the well-recognized trophic role of GABA signals that when blocked retards neuritic development and neuronal migration. Higher chloride concentrations and/or accumulation not only makes the actions of GABA, or glycine, depolarizing but also artificially prolongs the time course of the synaptic currents (Pitt et al., 2008; Houston et al., 2009). Thirdly, the developmental sequence of chloride cotransporters and the alterations of intracellular chloride concentrations attest to the importance of chloride in brain development. Fourth, at least in the hippocampus and other subcortical brain structures, GABAergic signaling develop before glutamatergic ones and conveys most if not all the activity at early developmental stages (Tyzio et al., 1999). Fifth, the developmental sequence of cortical networks involves initial intrinsic nonsynapse driven connectivity followed by synapse-driven patterns that entrain large neuronal populations; GABA signals play a crucial role in these developments, strongly suggesting that GABA plays different roles in immature and adult neurons. This is substantiated by both the observation that GABAergic interneurons follow a different migration route than excitatory/glutamatergic neurons, and by the demonstration that the radial migration of glutamatergic neurons requires operative GABA currents (Manent et al., 2005; Crepel et al., 2007; Bortone & Polleux, 2009). The biologic relevance of the GABA shift is illustrated by our recent discovery that oxytocin, which triggers delivery during pregnancy, also dramatically and abruptly reduces the intracellular levels of chloride in the brain of the newborn (Tyzio et al., 2006). This alteration transiently shifts GABA from excitation to inhibition and exerts a neuroprotective action on neurons by augmenting their resistance to anoxia during this vulnerable phase. Interestingly, following the observation that pain sensitivity was higher in C-section than in vaginally delivered newborns (Bergqvist et al., 2009), we reported an analgesic action of oxytocin and bumetanide in pups mediated by a direct reduction of intracellular chloride levels in pain pathways (Mazzuca et al., 2011), again reflecting the putative wide range applications of the developmental sequence of GABA actions. In a parallel study, using a dynamic two-photon imaging technique to determine the activity of several hundreds neurons, GABAergic interneurons were found to orchestrate the generation of GDPs, further confirming the crucial role of GABAergic signals (Bonifazi et al., 2009). Therefore, the developmental sequence of the polarity of GABA actions is not a curiosity made with in an

artificial in vitro preparation, but a convergence of a wide range of observations that bears relevance to a fundamental developmental mechanism.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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