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GABA action in immature neocortical neurons directly depends on the availability of ketone bodies

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Abstract

In the early postnatal period, energy metabolism in the suckling rodent brain relies to a large extent on metabolic pathways alternate to glucose such as the utilization of ketone bodies (KBs). However, how KBs affect neuronal excitability is not known. Using recordings of single NMDA and GABA-activated channels in neocortical pyramidal cells we studied the effects of KBs on the resting membrane potential (E_m) and reversal potential of GABA-induced anionic currents (E_{GABA}), respectively. We show that during postnatal development (P3–P19) if neocortical brain slices are adequately supplied with KBs, E_m and E_{GABA} are both maintained at negative levels of about −83 and −80 mV, respectively. Conversely, a KB deficiency causes a significant depolarization of both E_m (>5 mV) and E_{GABA} (>15 mV). The KB-mediated shift in E_{GABA} is largely determined by the interaction of the NKCC1 cotransporter and Cl^-/HCO_3 transporter(s). Therefore, by inducing a hyperpolarizing shift in E_m and modulating GABA signaling mode, KBs can efficiently control the excitability of neonatal cortical neurons.

Keywords: cortex, development, energy substrates, GABA, ketone bodies, resting potential.

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Depending on the physiological state, the brain is able to use different energy substrates including (but not limited to) the preferred substrates glucose, ketone bodies (KBs), and lactate (Lust *et al.* 2003; Nehlig, 1997; Prins 2008). Which metabolic substrates are used by the brain depends on the species, physiological demands, and the maturity of the enzymatic and transporter systems. For instance, while the newborn guinea pig brain mainly utilizes the products of glycolysis, KBs produced by ketogenesis provide an important energy source for the neonatal rat brain (Booth *et al.* 1980; Nehlig 2004). Indeed the immature rat brain utilizes KBs so effectively that they can represent about 30–70% of the total energy substrate pool (Nehlig 2004). KBs readily cross the blood–brain barrier in immature rodents [for review see Prins (2008) and Nehlig (2004)] and are present at high levels in CSF (Moore *et al.* 1976; Nehlig *et al.* 1991; Lust *et al.* 2003).

Ketone bodies, including acetoacetate, acetone, and the predominant KB in the blood 3-hydroxybutyrate (BHB) (Bough and Rho 2007) are produced by oxidation of fatty acids in the liver (Lockwood and Bailey 1970; Girard *et al.* 1992). In suckling rats, normal blood KB concentrations are about 1 mM (Ferre *et al.* 1978; Lockwood and Bailey 1971; Nehlig and Pereira de Vasconcelos, 1993; Page *et al.* 1971; Yeh and Zee 1976) – roughly 10 times higher than those found in adult rats (Hawkins *et al.* 1971; Nehlig and Pereira de Vasconcelos, 1993).

These observations suggest possible links between metabolic energy sources and neuronal functions in rodents in the early postnatal period. We investigated the dependence of some of the key neuronal properties that control the excitability of immature cortical pyramidal neurons,

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Abbreviations used: ACSF, artificial CSF; APs, action potentials; BHB, 3-hydroxybutyrate; BSA, bovine serum albumin; DIDS, Disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; DIOA, R(+)-Butylindazone, R-(+)-[(2-n-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid; EM, electron microscopy; KB, ketone bodies; KD, ketogenic diet; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; NMDAR, NMDA receptor; PBS, phosphate-buffered saline.

on the availability of KBs. In particular, this study focused on the relationship between KBs, E_m, and GABA signaling as GABA signaling is depolarizing and excitatory in the neonatal cortex *in vitro* [for review see Ben-Ari *et al.* (2007); Galanopoulou (2007); Kahle and Staley (2008)]. We found, however, that in neonatal cortical neurons in acute brain slices both the E_m level and GABA signaling mode correlate with the KBs level.

Materials and methods

Slice recordings

Brain slices were prepared from postnatal day P3 to P19 Swiss mice of both sexes. All animal protocols conformed to the French Public Health Service policy and the INSERM guidelines on the use of laboratory animals. Animals were rapidly decapitated and brains removed. Sagittal slices (300 μm) were cut using a tissue slicer (Microm International, Walldorf, Germany) in ice-cold oxygenated modified artificial CSF (ACSF) with 0.5 mM $CaCl_2$ and 7 mM $MgSO_4$, in which Na^+ was replaced by an equimolar concentration of choline. Slices were then transferred to oxygenated standard ACSF containing (in mM): 126 NaCl, 3.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 2.0 $CaCl_2$, and 10 D-glucose, pH 7.4, at room temperature (20–22°C) for at least 1 h before use. In the CO_2/HCO_3^--free solution HEPES (pH 7.4) replaced the bicarbonate in the standard ACSF, and the solution was bubbled with O_2. In the 5 mM bicarbonate solution, HEPES levels were adjusted accordingly. During recordings, slices were placed in a conventional fully submerged chamber superfused with ACSF (32–34°C). Pyramidal cells in the somatosensory and motor cortex were identified by the characteristic morphology of their soma, and the presence of a prominent apical dendrite using IR-differential interference contrast video microscopy. Pyramidal cells in both deep and superficial cortical layers were recorded. While the effects of BHB on cells in deep and superficial layers were qualitatively similar, no quantitative analysis of layer-dependent differences was performed. Patch-clamp recordings were performed using dual EPC-9 or EPC-10 amplifiers (HEKA Elektronik, Lambrecht/Pfalz, Germany). Pipettes (resistance of 3.5–8 MΩ) were pulled from borosilicate glass capillaries.

Patch-clamp recordings in cell-attached configuration

The currents through NMDA channels reversed close to 0 mV, therefore in cell-attached recordings, measurement of the reversal potential for NMDA receptor (NMDAR) channels gave the value for E_m [for more details see Tyzio *et al.* (2003)]. Meanwhile measurement of the reversal potential of the GABA receptor channels provided the driving force (DF_{GABA}) for the GABA-induced current [for more details see Rheims *et al.* (2008)].

Single channel recordings were performed using a pipette solution containing (in mM): (i) NaCl 140, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, GABA 0.01, pH adjusted to 7.3 with NaOH for recordings of single GABA channels and (ii) NaCl 140, KCl 2.5, $CaCl_2$ 2, HEPES 10, NMDA 0.01, glycine 0.01, pH adjusted to 7.3 with NaOH for recordings of single NMDA channels. Analysis of currents through single channels was performed using IGOR-Pro software (WaveMetrics Inc., Lake Oswego, OR, USA). All DF_{GABA} values were corrected for 2.1 mV (Tyzio *et al.* 2008). Patch clamp recordings in the whole-cell configuration were performed using the pipette solution (in mM): 115 potassium gluconate, 20 KCl, 4 ATP-Mg, 10 Na-phosphocreatine, 0.3 GTP-Na, and 10 HEPES; pH 7.3 adjusted by NaOH.

For recordings of action potentials (APs), cells were excited by random synaptic stimulation or by brief (200 ms) pressure applications of isoguvacine. A picospritzer (General Valve Corporation, Fairfield, NJ, USA) was used to puff-apply isoguvacine (30–100 μM in ACSF) from a glass pipette at a distance of about 100 μm from soma in recordings of APs. Gramicidin-D (50 μg/mL) perforated patch recordings were performed as described previously (Tyzio *et al.* 2003). Currents induced by a voltage ramp (from −100 to 0 mV, 1 s) in control were subtracted from those induced in the presence of isoguvacine (2 s applications). Because deviations in current amplitudes were considerable between different cells, in each case currents were normalized to the I–V slope at the reversal potential and averaged afterwards. During random synaptic stimulation, glutamatergic transmission was blocked by bath application of (2R)-amino-5-phosphonovaleric acid (40 μM) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) (5 μM), and nerve fibers were stimulated each 10 s via a bipolar metal extracellular electrode positioned in the white matter layer. Each stimulation sequence consisted of a random series of 0.2 ms stimuli (minimal interpulse interval 10 ms) during a period of 200 ms.

Immunohistochemistry at the light and electron microscopic level

We performed multiple immunofluorescence labeling at light microscopic and pre-embedding immunogold electron microscopic (EM) levels. P4 Wistar rats were deeply anesthetized by inhalation of isofluorane and transcardially perfused with a mixture of 4% paraformaldehyde and 0.1% of glutaraldehyde in 0.1 M sodium phosphate buffer. Brains were dissected out, post-fixed for 4–24 h in the same fixative solution, and stored in phosphate-buffered saline (PBS, 0.1 M, pH 7.4). Subsequently, specimens were coronally cut on a vibratome into slices of two different thicknesses: 50 μm for light microscopy or 120 μm for EM. Select sections were processed for floating immunohistochemistry. Sections were first treated for quenching with 0.05 M NH_4Cl in PBS for 20 min, then incubated for 2 h, at 20–22°C, with a mixture of 2.5% bovine serum albumin (BSA) with 2.5% normal goat serum in PBS. They were incubated for 24–48 h at 4°C with anti-KCC2 (rabbit; dilution 1 : 1000; US Biological, Euromedex, France), anti-neuron-specific nuclear protein (mouse neuronal nuclei; dilution 1 : 1000, Millipore Corporation, Bedford, MA, USA), and anti-cannabinoid receptor 1 antibodies (guinea pig; dilution 1 : 1000; gift of Dr. K. Mackie, Indiana University, Bloomington, IN, USA) diluted in PBS with BSA 0.5% and normal goat serum 0.5%.

For LM: after extensively rinsing in PBS, immunoreactivity was revealed by incubation for 2 h at 20–22°C with a carbocyanine-tagged secondary antibody (1 : 300; Jackson-Immunoresearch). Sections were finally analyzed using Zeiss (Jena, Germany) confocal microscopes (models: 510META and 710) with complementary software packages. Controls were routinely performed by omitting the primary antibodies.

For immunogold EM, after the primary KCC2 antibody, sections were post-fixed for 5 min in 4% paraformaldehyde and incubated

with a secondary 0.8 nm gold antibody (goat anti-rabbit, Aurion, Marne la Vallée, France), diluted 1 : 30 in PBS with 1% BSA at 4°C overnight. After repeated PBS + BSA rinses, sections were post-fixed (5 min) with 1% gluataraldehyde before a silver enhancement step (Aurion) for 1.5 h. Next, the specimens were osmicated in OsO_4, dehydrated in graded alcohol, and flat-embedded in Epon resin. Ultrathin sections (70 nm) of selected somatosensory cortical areas were cut with an ultracut microtome and visualized with a Zeiss 912 electron microscope. For the quantification of gold particles in the cytoplasm versus cell membrane, measurements were corrected for non-specific background labeling after counting the number of gold particles in an equivalent window.

Pharmacology

Drugs used were purchased from Tocris (Bristol, UK) (NBQX, D-(2R)-amino-5-phosphonovaleric acid) and Sigma (bumetanide and the racemic mixture of BHB; DL-3-hydroxybutyric acid sodium salt; St Louis, MO, USA). Within this racemic mixture, D-β-hydroxybutyrate is the primary mediator of the physiological effects of DL-BHB, and is the only form that can function as a substrate for mitochondrial BHB dehydrogenase (Klee and Sokoloff 1967; Passingham and Barton 1975; Robinson and Williamson 1980; Eaton *et al.* 2003). Consequently, only 50% of exogenous DL-BHB was expected to be utilized (Tsai *et al.* 2006).

Statistical analysis

Group measures were expressed as means ± SEM; error bars also indicated SEM. Statistical significance was assessed using the Wilcoxon's signed rank test, and the Mann–Whitney *U*-test. The level of significance was set at $p < 0.05$.

Results

Ketone bodies induce a hyperpolarizing shift in E_m and GABA signaling in neocortical pyramidal cells *in vitro*

Intracellular Cl^- concentration ($[Cl^-]_i$) and E_m are critical parameters controlling neuronal excitability. To measure these parameters noninvasively in pyramidal neurons, we performed cell-attached single channel recordings in neocortical slices (Tyzio *et al.* 2006; Rheims *et al.* 2008). To evaluate whether the mode of GABA action was inhibitory or excitatory, it was necessary to know the GABA current-

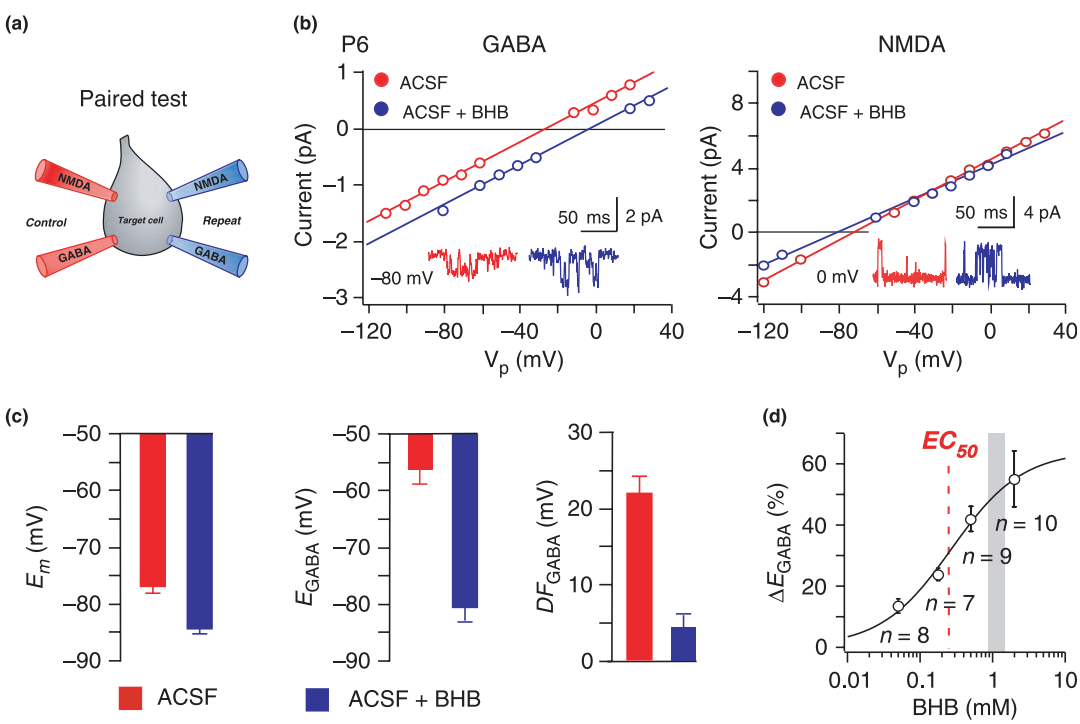

Fig. 1 3-β-Hydroxybutyrate induces a hyperpolarization of both the resting membrane potential and reversal potential of GABA-induced currents in neocortical neurons in slices. (a) Schematics of the generally used 'paired test' cell-attached single channel experimental protocol. (b) I–V relationships of currents through single $GABA_AR$ and NMDAR channels recorded in the same layer 2/3 pyramidal cell in a slice superfused with a standard ACSF and after addition of 4 mM DL-3-β-hydroxybutyrate (BHB, 4 mM of a racemic mixture; note that only D-isoform is the metabolically active one). (c) Summary plots of E_m, E_{GABA}, and DF_{GABA}, measured for an each individual neuron in control and in the presence of 4 mM BHB (P3–P8; $n = 28$, paired data). (d) A normalized concentration dependence on the effect of BHB on E_{GABA}. Data collected from P3 to P7 mice. As E_{GABA} in ACSF is strongly age-dependent (Rheims *et al.* 2008), the change in E_{GABA} obtained in the presence of BHB has been normalized to E_{GABA} in control ($E_{GABA}^{BHB} - E_{GABA}^{contr}/E_{GABA}^{contr}$) for each cell. The highlighted region depicts the physiological range of KBs levels in neonates.

induced deviations of membrane potential from the E_m level. Therefore, to obtain E_m in each intact neuron, we measured the reversal potential for NMDAR channels (Fig. 1a and b). In the same neuron, we also recorded $GABA_AR$ channels to assess the driving force for the GABA current (Fig. 1a and b) (DF_{GABA}, the difference between the reversal potential of the GABA-induced current, E_{GABA}, and the resting membrane potential E_m). These two values allowed us to calculate the reversal potential for the GABA current, E_{GABA} ($E_{GABA} = DF_{GABA} + E_m$) (Rheims *et al.* 2008; Tyzio *et al.* 2008). We supported energy metabolism by adding BHB, the predominant ketone body in the blood (Bough and Rho 2007), to the standard ACSF. A 'paired test' protocol was used (as illustrated in Fig. 1a and b). First, NMDAR and $GABA_AR$ channels were recorded on a neuron. Subsequently, following at least 40 min wash-in and incubation in KB-containing ACSF, single NMDAR and GABA receptor channel recordings were repeated on the same target neurons.

In all neocortical pyramidal neurons ($n = 28$) from P3 to P8 mice, 4 mM BHB (4 mM of a racemic mixture; only the D-isoform was metabolically active) induced a significant hyperpolarization of E_m (-76.9 ± 1.25 mV in ACSF; -84.5 ± 0.87 mV in ACSF + BHB, $p < 10^{-4}$; Fig. 1c). In slices superfused with standard ACSF, the action of GABA was strongly depolarizing (E_{GABA} was -56.4 ± 2.3 mV; Fig. 1c), as has been reported in previous studies (Rheims *et al.* 2008; Owens *et al.* 1996a, Yamada *et al.* 2004; Cancedda *et al.* 2007). However, BHB induced a large negative shift in E_{GABA} (to -80.1 ± 2.6 mV) in all these neurons. Figure 1d shows the concentration dependence of this BHB effect on E_{GABA} in P3–P7 mice. As E_{GABA} in the control standard ACSF was strongly age-dependent (Rheims *et al.* 2008), the change in E_{GABA} obtained in the presence of BHB was normalized to E_{GABA} in control ($E_{GABA}^{BHB} - E_{GABA}^{contr}/E_{GABA}^{contr}$) for each cell. The EC_{50} was ~0.3 mM, which was lower than physiological BHB concentrations in neonatal rats (Lockwood and Bailey 1971; Page *et al.* 1971; Yeh and Zee 1976; Ferre *et al.* 1978).

As an alternate method to measure the changes in E_{GABA} we utilized the gramicidin perforated patch technique, regularly used to estimate $[Cl^-]_i$ [e.g., Owens et al. (1996), Yamada *et al.* 2004, and Cancedda *et al.* 2007)]. Similarly to the results obtained with single channel recordings, BHB induced a negative shift in E_{GABA} (Fig. 2a) from -62.5 ± 3.2 mV to -82.9 ± 3.1 mV ($n = 5$). In accordance with these observations the excitatory action of GABA on neocortical pyramidal cells was strongly reduced by BHB. Figure 2b shows that following stimulation of nerve fibers by short random pulse trains (Rheims *et al.* 2008) GABA synaptic transmission (glutamatergic transmission was blocked by (2R)-amino-5-phosphonovaleric acid and NBQX) reliably triggered APs in neurons (Fig. 2a,b; cell-attached configuration). After addition of BHB, in most cases the same stimulation failed to initiate APs in the same pyramidal cells. The recorded neurons, however, readily generated APs in response to the stimulation in the whole-cell configuration with 20 mM Cl^- in the pipette solution ($E_{GABA} \approx -49$ mV; Fig. 2b) verifying that the intrinsic neuronal properties remained stable following the ~40 min of the solution exchange procedure. Similar effects were also obtained by puff application of isoguvacine, a $GABA_AR$ agonist (Fig. 2c).

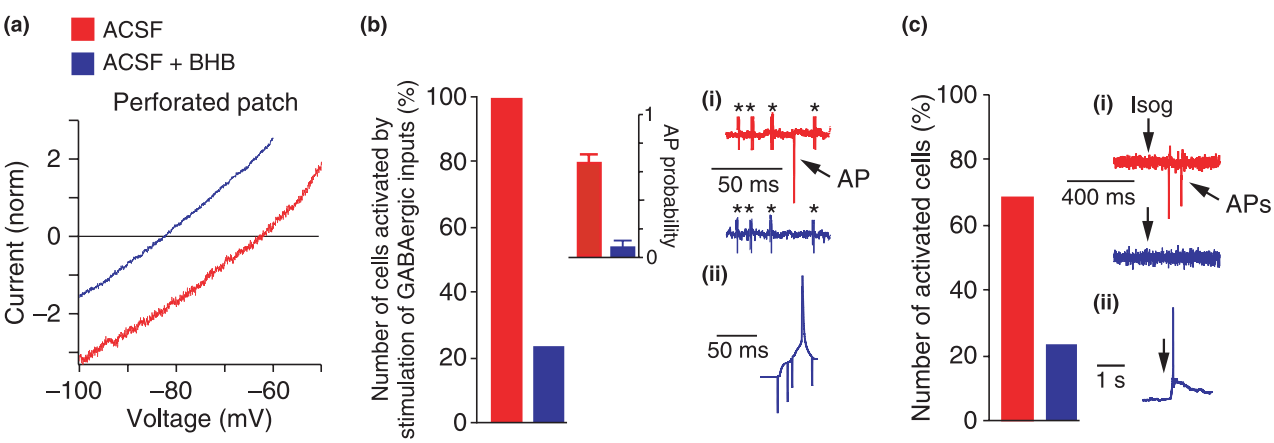

Fig. 2 Alternative methods reveal hyperpolarization of E_{GABA} induced by BHB. (a) Averaged I–V relationships of normalized puff-isoguvacine (Isog)-induced currents (five cells in control and five cells in BHB) obtained by voltage ramps during gramicidin perforated-patch recordings. (b) Extracellular stimulation of nerve fibers by short pulse (asterisks) trains in the presence of the glutamate ionotropic receptors antagonists, NBQX (5 μM) and APV (40 μM), reliably induced APs in pyramidal cells in ACSF [(i) in red, cell-attached recordings] but did not in the presence of 4 mM BHB [(i) in blue, the same pyramidal cell]. However, APs were readily initiated in the same cells in the whole-cell configuration [(ii), 20 mM $[Cl^-]_i$]. (c) The proportion of cells excited by brief applications of Isog in control and in the presence of 4 mM BHB. Similarly to synaptic stimulation, neurons did not generate APs in the presence of BHB during cell-attached recordings (i) but reliably fired in the whole-cell configuration (b). Color coding (ii, top) is the same for all figures.

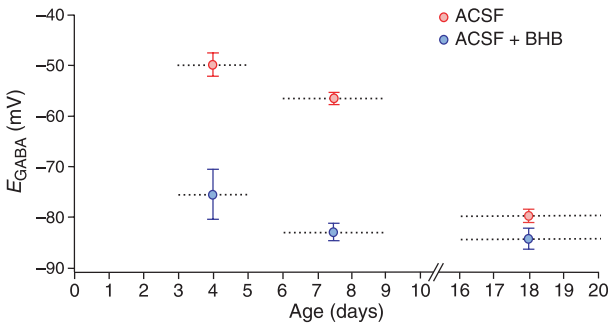

Fig. 3 Ketone bodies induce a hyperpolarizing shift in GABA action *in vitro* during early postnatal weeks. Summary of E_{GABA} values obtained either in the absence or presence of KBs. Dotted lines indicate the age range. The BHB-mediated change in E_{GABA} was significant in all age groups ($p < 0.02$ in all cases).

Therefore, supplementation of the standard ACSF with the endogenous energy substrate BHB induced a negative shift in E_m, decreased the positive driving force for GABA-induced currents, and strongly reduced the excitatory GABA action in neonatal pyramidal neurons.

Figure 3 summarizes the E_{GABA} values obtained using the cell-attached single channel recording method, above, in different age groups. While the values of E_{GABA} measured in standard ACSF are in agreement with the well-documented E_{GABA} developmental profile (Rheims *et al.* 2008; Owens *et al.* 1996a, Yamada *et al.* 2004; Cancedda *et al.* 2007), the E_{GABA} values in the presence of BHB displayed similarly negative values throughout the developmental period studied. (E_{GABA} values in the different age groups were not significantly different, $p > 0.1$, unpaired *t*-test). These results suggest that in the presence of KBs neuronal $[Cl^-]_i$ was low during postnatal development, and GABA signaling was inhibitory.

KB-dependent mechanism of $[Cl^-]_i$ regulation

Several transporter families are involved in maintaining neuronal Cl^- homeostasis (Gamba 2005; Farrant and Kaila 2007), with the chloride cation cotransporters, KCC2 and NKCC1, considered to be the main contributors to $[Cl^-]_i$ regulation in cortical neurons (Farrant and Kaila 2007). While NKCC1 pumps Cl^- into a cell, KCC2 is responsible for Cl^- extrusion. An additional group, the bicarbonate chloride exchangers and transporter family, are also able to modulate $[Cl^-]_i$ (Romero *et al.* 2004; Pushkin and Kurtz 2006), although their role as regulators of intracellular pH regulators has received more attention (Chen *et al.* 2008). This suggests three main candidates which could mediate the KB effect on E_{GABA}: KCC2, NKCC1, and the bicarbonate chloride exchangers.

As a major neuronal Cl^- extruder, KCC2 is a likely candidate for the changes observed in E_{GABA}. However, immunohistochemistry suggested that KCC2 was localized to the cytoplasm as well as close to the cell membrane. This was confirmed by EM immunogold analysis. At this age (P4) the majority of transporter molecules (87 ± 4%, $n = 19$ cells) were not distributed to the membrane but were still located in cytoplasm of neocortical pyramidal cells (Figs. 4a and b) suggesting that KCC2 does not play a major role in the effect we observed. Electrophysiological data confirmed that KCC2 was not significantly involved in the KB-dependent regulation of $[Cl^-]_i$. Indeed, blockade of KCC2 by the antagonist R(+)-Butylindazone, R-(+)-[(2-n-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid (DIOA) (Pond *et al.* 2004) did not induce a significant change in E_{GABA} (P7–P8; −60.7 ± 2.2 mV in ACSF, $n = 13$, −63 ± 2.4 mV in ACSF + DIOA, $n = 8$, $p > 0.5$; Fig. 4c). Additionally, BHB still induced its effect on E_{GABA} in the presence of DIOA (P5-P8; −62.96 ± 2.36 in DIOA $n = 8$, −80.4 ± 6.1 mV in ACSF + BHB + DIOA, $n = 7$, $p < 0.05$; Fig. 4c). As a positive control we tested the efficacy of DIOA on P15 neurons, where a low $[Cl^-]_i$ (Yamada *et al.* 2004; Rheims *et al.* 2008) was maintained by the action of KCC2 (Farrant and Kaila 2007). In this older age group blockade of KCC2 by DIOA induced significant depolarization of E_{GABA} (−81,5 ± 1.8 mV in ACSF (P14–19), $n = 25$, −68.1 ± 4.4 mV in ACSF + DIOA, $n = 5$, $p < 0.05$) verifying the effectiveness of the drug.

The second major group of neuronal Cl^- extruders, the bicarbonate-chloride exchangers, have an absolute requirement for the presence of HCO_3^- in the extracellular media (Romero *et al.* 2004; Pushkin and Kurtz 2006). We therefore performed experiments using HCO_3^--free solution (see Materials and methods). This solution did not significantly affect the depolarizing E_{GABA} observed using standard ACSF (P6–P7; −52.1 ± 3.7 mV in ACSF, $n = 10$, −56.9 ± 2.7 mV in HCO_3^--free solution, $n = 9$; $p > 0.7$; Fig. 4d). However, it did prevent the hyperpolarizing effects of BHB on E_{GABA} (P6–P8; −56.9 ± 2.7 mV in HCO_3^--free solution, $n = 9$; −56.8 ± 3.1 mV in HCO_3^--free + BHB; $n = 9$; $p > 0.9$) (Fig. 4d). Partial activation of the bicarbonate chloride exchangers by the addition of 5 mM HCO_3^- to the extracellular solution significantly increased BHB's effect on E_{GABA}, to ∼50% of the BHB effect in standard ACSF (26 mM HCO_3) (P5–P6; −51.7 ± 1.3 mV in ACSF, $n = 11$, −72.1 ± 3.1 mV in 5 mM HCO_3^- + BHB, $n = 8$; $p < 10^{-4}$) (Fig. 4d). Finally, DIDS, an antagonist of anionic exchangers blocked the effect of BHB on E_{GABA} [−57.4 ± 2.3 mV disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) alone, $n = 6$; −59.8 ± 2.7 mV DIDS + BHB, $n = 9$; $p > 0.5$ (Fig. 4e)].

The strongly depolarized E_{GABA} observed in the HCO_3^--free solution, also observed in standard ACSF (Rheims *et al.* 2008), was mainly because of the activity of the NKCC1 cotransporter. Similar to previous observations using standard ACSF (Rheims *et al.* 2008) the application of the NKCC1 selective antagonist, bumetanide (10 μM), induced a large hyperpolarizing shift in E_{GABA} (to

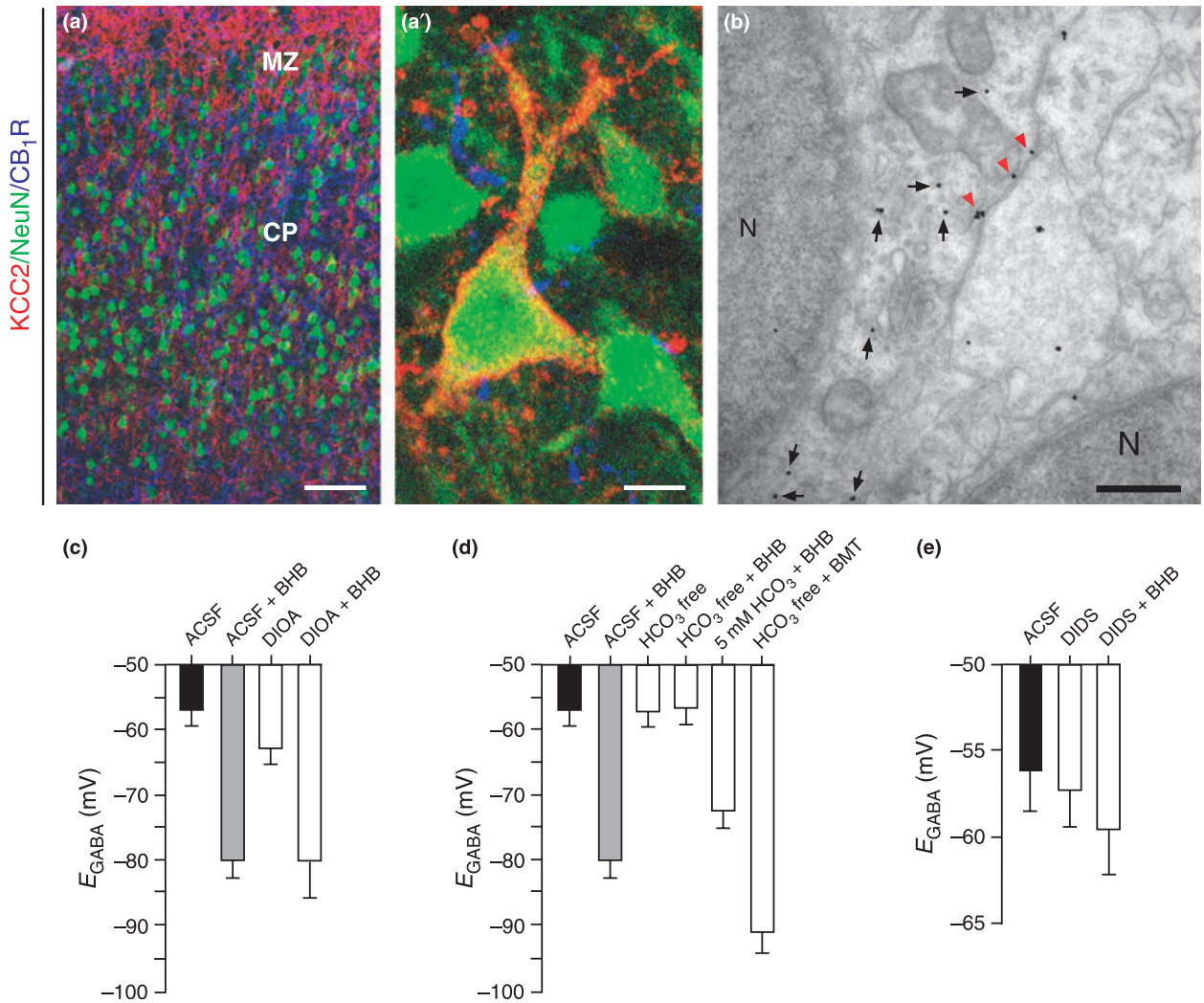

Fig. 4 Mutual activity of chloride-bicarbonate transporter(s) and NKCC1 underlies the changes in $[Cl^-]_i$. **(a)** Localization of KCC2 in neonatal neocortical neurons. KCC2 is present in the cortex at P4 and it is post-synaptic (dendritic) as it labels the apical tuft of pyramids and does not colocalize with CB1Rs (blue) that are known to be pre-synaptic markers. MZ = marginal zone, CP = cortical plate, NeuN = anti-neuron-specific nuclear protein marker. (a′) High resolution image showing KCC2 in the cell cytoplasm and in the vicinity of the cell membrane. (b) Immunogold electron microscopy shows that KCC2 is predominantly localized in the cytoplasm (black arrows) although a few gold particles localize near the cytoplasmic membrane (red arrow heads). N = nucleus, Scale bars for a(i) = 50 μm, a(ii) = 5 μm, b = 0.5 μm. (c) Blockade of KCC2 by DIOA does not prevent the action of BHB. (d) The presence of HCO_3^- in the extracellular solution is necessary for the effect of BHB on E_{GABA}. High $[Cl^-]_i$ in the HCO_3^--free solution is because of activity of NKCC1 (BMT, bumetanide, a selective antagonist of NKCC1). (e) BHB does not affect E_{GABA} under blockade of Cl^-/HCO_3^- transporters by DIDS. N.B.: In c–e data were obtained in independent experiments (not using the 'paired test' protocol). For comparison E_{GABA} values obtained in ACSF and/or ACSF + BHB (from Fig. 1) are also shown.

−91 ± 3 mV, $n = 8$; Fig. 4d). These results suggest that the KB-mediated changes in $[Cl^-]_i$ we observed arise as a result of a shift in the balance of activity of NKCC1 and bicarbonate-chloride exchanger(s).

Discussion

In this study, we show that during the postnatal development of the neocortex, two critical parameters, E_m and E_{GABA}, are affected by the availability of KBs. KBs effectively reduce the depolarizing GABA action in a dose-dependent manner. We suggest that this KB-dependent control of GABA action forms part of a system, which links the signaling and metabolic aspects of neuronal excitability.

Previous studies *in vitro* have reported that GABA signaling in neonates is depolarizing and excitatory [for reviews see Ben-Ari (2002); Ben-Ari *et al.* (2007); Galanopoulou (2007); Kahle and Staley (2008)]. Meanwhile, our data obtained in

slices showed that in the presence of KBs, values of E_{GABA} in neocortical pyramidal neurons were close to E_m, and did not change significantly during postnatal development, being maintained at about −80 mV (see Fig. 3). We cannot exclude the possibility that these values may differ in dendritic (Gulledge and Stuart 2003) or axonal (Price and Trussell 2006; Trigo *et al.* 2007; Khirug *et al.* 2008) compartments, an issue for future studies. Additionally, in this study we have limited our investigations to pyramidal cells, and the effects of KBs on interneurons remain to be explored.

Nevertheless, the present observations suggest that energy substrates in the developing brain are an important issue to consider when studying neonatal neuronal excitability. Indeed, the most straightforward explanation for the difference between the results of the current study and those of previous studies of the development of neonatal GABA signaling lies in the fact that the brain of the suckling rodent relies strongly on KBs (Cremer and Heath 1974; Dombrowski *et al.* 1989; Hawkins *et al.* 1971; Lockwood and Bailey 1971; Lust *et al.* 2003; Page *et al.* 1971; Pereira de Vasconcelos and Nehlig 1987; Schroeder *et al.* 1991; Yeh and Zee 1976). Glucose utilization is limited at this age (Dombrowski *et al.* 1989; Nehlig, 1997; Nehlig *et al.* 1988; Prins 2008) because of the delayed maturation of the glycolytic enzymatic system (Dombrowski *et al.* 1989; Land *et al.* 1977; Leong and Clark 1984; Prins 2008). Use of glucose as the sole energy substrate caused an increase in neonatal neuronal $[Cl^-]_i$ in our experiments, similar to that observed previously, while the addition of KBs resulted in a hyperpolarizing shift in both E_m and E_{GABA}. These results highlighted the need for caution in the interpretation of results obtained from neonatal brain slices superfused with standard ACSF.

The cation chloride cotransporters NKCC1 and KCC2 have been suggested to be the main regulators of neuronal Cl^- homeostasis both during development (Farrant and Kaila 2007; Fiumelli and Woodin 2007) and in pathology (Galanopoulou, 2007; Kahle and Staley, 2008; Kahle *et al.* 2008). Although the possible contribution of anion exchangers to neuronal Cl^- homeostasis has been noted previously (Farrant and Kaila 2007; Hentschke *et al.* 2006; Hubner *et al.* 2004; Pfeffer *et al.* 2009), they have not attracted the same degree of attention. Results from our study demonstrate, however, that the Cl^-/HCO^-_3 transporter system is strongly involved in the KB-mediated regulation of $[Cl^-]_i$ during postnatal development. Within this family, the Na-dependent Cl^-/HCO^-_3 transporter (NDCBE), is of particular interest as it is expressed in the cortex (Chen *et al.* 2008) and has a strong dependence on ATP for its action (Chen *et al.* 2008; Davis *et al.* 2008; Romero *et al.* 2004). In addition, the sodium-driven chloride bicarbonate exchanger (NCBE), (Giffard *et al.* 2003; Hubner *et al.* 2004; Lee *et al.* 2006) was expressed in the brain early during prenatal development and its expression preceded that of KCC2 (Hubner *et al.* 2004).

In neonatal neocortical neurons the interaction of NKCC1 and the Cl^-/HCO_3^- transporter(s) maintained $[Cl^-]_i$, with KCC2 playing a less significant role at this stage. In the absence of KBs, when Cl^-/HCO_3^- transporter(s) were less effective, the role of NKCC1 as a Cl^- loader was especially noticeable and resulted in a depolarizing E_{GABA}. During development the contribution of KCC2 to neocortical neuronal Cl^- homeostasis is likely to increase (Stein *et al.* 2004; Zhang *et al.* 2006), and the balance between the actions of the different Cl^- transporters in adults should be studied in the future.

In humans, blood levels of KBs increase considerably during fasting, strenuous exercise, stress, or on the high-fat, low-carbohydrate ketogenic diet (KD) (Newburgh and Marsh 1920). A rapidly growing body of evidence indicates that the KD can have numerous neuroprotective effects (Gasior *et al.* 2006). During treatment with the KD, levels of KBs increase in both blood and brain, and cerebral metabolism adapts to preferentially use KBs as an alternate energy substrate to glucose (Kim do and Rho 2008). In children, the KD has been used as an effective treatment for medically refractory epilepsy (Freeman *et al.* 2007; Hartman and Vining 2007). However, despite nearly a century of use, the mechanisms underlying its clinical efficacy have proved elusive (Morris 2005; Bough and Rho 2007; Kim do and Rho 2008). Suckling rodents provide a natural model of the KD because of the high ketogenic ratio (Wilder and Winter 1922) of rodent milk (Page *et al.* 1971; Nehlig 1999). We propose that the KB-induced modulation of GABA-signaling may constitute a mechanism of anticonvulsive actions of the KD.

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