

# ANOTHER LOOK AT EARLY GABAergic NEUROTRANSMISSION: MAYBE IT'S NOT SO EXCITING AFTER ALL!

**GABA Action in Immature Neocortical Neurons Directly Depends on the Availability of Ketone Bodies.** Rheims S, Holmgren CD, Chazal G, Mulder J, Harkany T, Zilberter T, Zilberter Y. *J Neurochem* 2009;110(4):1330–1338. 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.

**Energy Substrate Availability as a Determinant of Neuronal Resting Potential, GABA Signaling and Spontaneous Network Activity in the Neonatal Cortex In Vitro.** Holmgren CD, Mukhtarov M, Malkov AE, Popova IY, Bregestovski P, Zilberter Y. *J Neurochem* 2010;112(4):900–912. While the ultimate dependence of brain function on its energy supply is evident, how basic neuronal parameters and network activity respond to energy metabolism deviations is unresolved. The resting membrane potential ( $E_m$ ) and reversal potential of GABA-induced anionic currents ( $E_{GABA}$ ) are among the most fundamental parameters controlling neuronal excitability. However, alterations of  $E_m$  and  $E_{GABA}$  under conditions of metabolic stress are not sufficiently documented, although it is well known that metabolic crisis may lead to neuronal hyper-excitability and aberrant neuronal network activities. In this work, we show that in slices, availability of energy substrates determines whether GABA signaling displays an inhibitory or excitatory mode, both in neonatal neocortex and hippocampus. We demonstrate that in the neonatal brain,  $E_m$  and  $E_{GABA}$  strongly depend on composition of the energy substrate pool. Complementing glucose with ketone bodies, pyruvate or lactate resulted in a significant hyperpolarization of both  $E_m$  and  $E_{GABA}$ , and induced a radical shift in the mode of GABAergic synaptic transmission towards network inhibition. Generation of giant depolarizing potentials, currently regarded as the hallmark of spontaneous neonatal network activity *in vitro*, was strongly inhibited both in neocortex and hippocampus in the energy substrate enriched solution. Based on these results we suggest the composition of the artificial cerebrospinal fluid, which bears a closer resemblance to the *in vivo* energy substrate pool. Our results suggest that energy deficits induce unfavorable changes in  $E_m$  and  $E_{GABA}$ , leading to neuronal hyperactivity that may initiate a cascade of pathological events.

## COMMENTARY

Within the past 20 years, the notion that GABAergic excitation plays a significant role in the developing

brain has been accepted as dogma in scientific circles—a viewpoint based on an abundance of studies consistently demonstrating a GABA-induced depolarizing response in immature neocortical and hippocampal neurons (1,2). This paradoxical response is then thought to quickly revert to the mature phenotype of cellular membrane hyperpolarization. The primary mechanism underlying the developmental switch in GABAergic

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neurotransmission is a consequence of the differential expression and activity of two cation chloride cotransporters, sodium potassium chloride cotransporter 1 (NKCC1) and potassium chloride cotransporter 2 (KCC2), which import and extrude chloride, respectively (3). Further, GABA-mediated excitation is believed to contribute to giant depolarizing potentials, which have been considered a hallmark of spontaneous network activity in the immature brain (4). Traditionally, these concepts have been invoked as critical factors underlying the enhanced seizure propensity of the developing brain, in both animal models as well as surgically resected human epileptic tissues (5).

The most compelling experimental evidence for GABA-evoked excitation has come from in vitro electrophysiological studies in acute brain slices that have employed glucose in artificial CSF (ACSF) (2). While glucose has been shown to be necessary for brain slice viability, the standard composition of ACSF does not fully mirror in vivo metabolic conditions, especially in the immature brain. Specifically, there is a notable absence of other important energy substrates, such as ketone bodies (e.g.,  $\beta$ -hydroxybutyrate [BHB], lactate, and pyruvate), which are highly relevant to developing neurons and glia during the time when the metabolic glycolytic machinery is immature (6–8).

It is in this context that Rheims et al. and Holmgren and colleagues, in two provocative studies, evaluated the effects of metabolic energy substrates on GABA-induced depolarization in immature neurons of both neocortical and hippocampal tissue, utilizing complementary investigative techniques. In the study by Rheims et al., the investigators found that a physiologically relevant concentration of BHB (2 mM of the D-isomer in a racemic mixture), when used with standard 10 mM D-glucose, induced a hyperpolarizing shift in the resting membrane potential ( $E_m$ ) of immature neocortical pyramidal neurons from Swiss mice, aged postnatal (P) days 4–8, under both cell-attached single-channel and gramicidin-perforated patch recording conditions (the latter, importantly, prevents dialysis of intracellular contents seen with whole-cell recordings). The GABA reversal potential ( $E_{GABA}$ ) was also maintained in a hyperpolarized range similar to what was seen in older P18 animals ( $-82.9 \pm 2.1$  mV vs  $-62.5 \pm 3.2$  mV) when incubated with BHB. Conversely, in the absence of BHB, the expected depolarized  $E_m$  and  $E_{GABA}$  values were seen in younger aged brain slices.

To assess whether these findings were a result of changes in the major chloride extruder, KCC2, Rheims and colleagues then studied the localization of this cotransporter using immunocytochemical and electron microscopic immunogold techniques. They found that the bulk of KCC2 molecules were localized to the cytoplasm (and not plasma membrane) of neocortical pyramidal neurons, suggesting that KCC2 was not significantly involved in the acute ketone-body-mediated effect. To

further confirm this notion, they found that pharmacological blockade of KCC2 did not induce significant changes in  $E_{GABA}$ . Interestingly, use of  $\text{HCO}_3^-$ -free ACSF prevented the hyperpolarizing effects of BHB on  $E_{GABA}$ , suggesting that the  $\text{HCO}_3^-$ -dependent bicarbonate–chloride exchanger (representing the second major group of neuronal chloride extruders) was prominently involved (9).

In a subsequent study, Holmgren and colleagues extended these findings and asked whether: 1) other developmentally important energy substrates, such as lactate and pyruvate, might also affect GABA-induced membrane excitation; 2) similar observations could be made in neonatal hippocampus; and 3) metabolic substrates might affect the incidence of giant depolarizing potentials, as measured using field recording techniques. Indeed, these investigators found that supplementing standard ACSF with either BHB, pyruvate, or lactate resulted in hyperpolarizing shifts in both  $E_m$  and  $E_{GABA}$  in neocortical as well as hippocampal pyramidal neurons from Swiss mice or Wistar rats, aged P3–P8, of both sexes. Moreover, they were unable to detect giant depolarizing potentials in either neocortical or hippocampal networks in immature mice or rats, aged P4–P6, when the ACSF was supplemented by these metabolic substrates. Importantly, since acidification might explain the hyperpolarizing effects of the substrates, they measured the intracellular pH using a fluorescent pH-sensitive dye and found that energy substrate enrichment of ACSF led to a small and insignificant pH change (a decrease of only 0.05 of a unit).

Collectively, these studies cast doubt on the biological relevance of GABA-induced depolarization as evidenced by a multitude of cellular electrophysiological studies. Certainly, these authors make a compelling case for a thoughtful re-examination of the time-honored use of ACSF formulations that solely employ glucose as an energy substrate. This research has important implications for the phenomenon of increased seizure susceptibility of the immature brain and also for GABA excitation as a trophic mechanism during early brain development.

However, in spite of the apparent consistency in their experimental results, Rheims et al. and Holmgren and colleagues need to reconcile their data with other morphological studies demonstrating altered expression of cation chloride cotransporters NKCC1 and KCC2 without the potential confound of standard ACSF, especially since these cotransporters are considered the major determinants of the transmembrane electrochemical gradient for the chloride ion (3,5). Intriguing as their findings are, the authors have not yet firmly established a mechanism for their general observation of metabolic substrate-induced reversal of GABA excitation, despite preliminary evidence invoking the bicarbonate–chloride exchanger. Nevertheless, if the observations of Rheims et al. and Holmgren and colleagues are ultimately validated, then a couple of generations

of in vitro studies are likely to be at risk for relegation to the murky domain of artifact.

One additional suggestion made by these authors bears further comment. Do their findings relate in any meaningful way to the mechanism of action of the ketogenic diet—an established treatment for medically refractory epilepsy (10)? Although ketonemia is prominent with this treatment, whether ketone bodies themselves mediate the ketogenic diet's anticonvulsant effect remains controversial, as blood ketone levels do not correlate tightly with seizure control in either animal models or humans with intractable epilepsy. With respect to BHB, the major ketone body, there are, as yet, no data demonstrating a direct anticonvulsant effect. Although Rheims et al. and Holmgren and colleagues indicate that their results may be similar to the mechanism of ketogenic action, this link remains speculative at best.

Ketone bodies, in particular, have previously been shown to be neuroprotective, likely through enhanced ATP production and a decrease in reactive oxygen species (11). Are the effects observed by the Rheims et al. and Holmgren and colleagues merely a downstream action of enhanced ATP levels (and as such, an indication of better preservation of important homeostatic mechanisms, such as the Na<sup>+</sup>, K<sup>+</sup>-ATPase) and/or modulation of ion channels by reactive oxygen species? How would such actions modulate the activity of specific transporters or exchangers? Additionally, do metabolic substrates, such as ketones, lactate, and pyruvate, affect the expression of the bicarbonate–chloride exchanger (only indirect evidence was provided by these studies)? What would the effect of such metabolic substrates on  $E_m$  and  $E_{GABA}$  be in the epileptic (i.e., not normal) brain? Clearly, there are important ramifications of their work, but many questions remain outstanding.

It should not be surprising that maintenance of a healthy metabolic pool would enhance normal mature neuronal function. However, whether GABA-evoked depolarization is merely

a developmental aberration that is compensated for by differential and age-dependent utilization of energy substrates or whether it is still a fundamental physiological phenomenon important for neuronal maturation, and possibly seizure genesis, remains unclear.

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