Khakhalin AS. Questioning the depolarizing effects of GABA during early brain development. J Neurophysiol 106: 1065–1067, 2011. First published May 18, 2011; doi:10.1152/jn.00293.2011.— During early brain development, γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the adult brain, has been thought to be an important source of excitatory neurotransmission. This view, however, was recently challenged by a series of studies that claim that the excitatory effect of GABA is due to non-physiological in vitro experimental conditions. In this article, we aim to summarize results that support and challenge the traditional point of view, and indicate some strong and weak points of both positions.

For over 20 years it has been generally accepted that γ-aminobutyric acid (GABA), which serves as the key inhibitory neurotransmitter in adult brains, may have an excitatory effect during early brain development. Immature neural cells typically express a different set of membrane ionic transporters than adults, which creates a high concentration of chloride ions inside the cell. This causes a depolarizing shift of the reversal potential for chloride, and ultimately results in GABA-mediated postsynaptic currents that are depolarizing, and even spike generating. One important manifestation of GABA-mediated excitation is in the form of slow-correlated waves of depolarization known as giant depolarizing potentials (GDPs). This excitatory action of GABA is thought to be important for the maturation of neural networks, allowing newly formed silent synapses that lack AMPA receptors on their postsynaptic membranes to be depolarized for the first time and express NMDA receptor-mediated Hebbian-type plasticity (Ben-Ari et al. 2007).

Recently, however, this concept was challenged in a series of studies by Zilberter and colleagues, who were motivated by the fact that the overwhelming majority of evidence for the depolarizing action of GABA had been obtained from various in vitro preparations. These new studies argue that a relatively modest change in experimental conditions, such that they better reproduce in vivo conditions, abolishes the depolarizing action of GABA in immature neural networks (Holmgren et al. 2010; Rheims et al. 2009). Namely, they supplemented the artificial cerebro-spinal fluid (ACSF), in which the in vitro preparation is immersed and which typically only uses glucose as a sole energy source, with alternative energy sources, such as the ketone body β-hydroxybutyric acid (BHB), lactate, and pyruvate. These energy sources are known to be present in millimolar concentrations in extracellular fluid of developing brains (Zilberter et al. 2010). They found that for all alternative ACSF formulations used, the action of GABA in slices from immature brains became more hyperpolarizing and adult-like (Holmgren et al. 2010; Rheims et al. 2009), and the GDPs were reported to become less frequent or disappeared altogether (Holmgren et al. 2010). The change in GABA action was attributed to the relative inability of immature neurons to metabolize glucose efficiently, in contrast to BHB, lactate, or pyruvate (Zilberter et al. 2010). It was suggested that in the absence of alternative energy sources, cellular homeostasis is compromised in immature neurons, and certain accessory processes (namely the transport of chloride out of the cell) are sacrificed in favor of more vital functions. This would imply that the depolarizing action of GABA, as observed in glucose-based ACSF, is not a physiologically relevant phenomenon, but rather an artifact of insufficient energy supply (Holmgren et al. 2010).

These studies by Zilberter and colleagues naturally aroused a great deal of controversy, and several research groups independently endeavored to verify and reinterpret them; the summary of these attempts is shown in Fig. 1. It was quickly noticed that all three substances used as alternative energy sources also belong to a class of weak organic acids that are known to induce intracellular acidification, which made it possible to ascribe the effect of alternative ACSF solutions on GABA action to a drop in intracellular pH (Ruusuvuori et al. 2010). In this article, we attempt to break down this controversy by considering experimental evidence supporting each side of this argument, trying at each stage to differentiate between the results reported and the interpretation of these results.

Do weak acids change action of GABA in immature brain preparations, making it more adult-like? The effect of weak acids on GABA reversal potential and GDP generation was initially described for 4–5 mM concentrations of BHB (Rheims et al. 2009) and lactate and pyruvate (Holmgren et al. 2010), and was later confirmed by independent research groups for similar concentrations of pyruvate (Tyzio et al. 2011), lactate and propionate (Ruusuvuori et al. 2010). When ACSF was enhanced with weak acids, GABA transmission in slices from early postnatal rats and mice was found to be hyperpolarizing, as in slices from adult animals, and the GDPs were not generated. The effect of 4–5 mM of BHB, however, was not reproduced by other groups (Kirmse et al. 2010; Tyzio et al. 2011), and it was suggested that the BHB used for the initial experiments of Rheims et al. (2009) might have been chemically contaminated, as it was shown that at least some batches of this chemical from some suppliers (although not from those reportedly used by Rheims et al.) contain dibenzylamine, a...
substance able to affect GABA reversal potential even if applied in micromolar concentrations (Tyzio et al. 2011). In one study (Kirmse et al. 2010), the effect of pyruvate was not reproduced as well, but this difference may be attributed to the use of younger animals [mice at postnatal days 1–4 (in Kirmse et al. 2010), as opposed to rats at postnatal days 4–8, as used in the majority of experiments]. It is known that around birth, GABA temporarily becomes strongly hyperpolarizing, and then shifts back to a more depolarizing action in a time frame of 1–2 days, thus making the population of postnatal days 1–4 mice highly heterogeneous in respect to GABA action, and prone to pooling error (Tyzio et al. 2011). In summary, at least for 4–5 mM concentrations of lactate and pyruvate, the effect seems to be reproducible (see Fig. 1A for a summary of observed effects).

Are the 4–5 mM concentrations of weak acids in ACSF physiological? In a recent publication (Tyzio et al. 2011), Ben-Ari and colleagues criticize the experiments of Zilberter and colleagues for using relatively high 4–7 mM concentrations of weak acids in ACSF (Holmgren et al. 2010). Actual total concentrations of lactate and pyruvate in newly born rat pups’ blood plasma were reported to be only 1.6 mM (Tyzio et al. 2011), making the physiological relevance of using a ~5 mM weak acid concentration questionable. This comparison may be not valid, however, as it is well known from microdialysis studies that the extracellular fluid, immediately surrounding neural cells, differs in its composition not only from the blood plasma, but even from the cerebrospinal fluid. In particular, concentration of lactate in the extracellular fluid of rats and humans was found to be 2–5 times higher than in the blood plasma (Zilberter et al. 2010). The reason for this difference is thought to be in the specifics of metabolic support and glutamate transport provided to neurons by astrocytes (Pellerin et al. 2007), and it is this fine neural-glial interaction that can be destroyed in vitro during a typical brain preparation (Holmgren et al. 2010; Zilberter et al. 2010). If applied to results of blood plasma measurements reported by Tyzio et al. (2011), this known ratio of concentrations gives an estimation of ~5 mM lactate+pyruvate in the extracellular fluid in the brains of early postnatal rat pups, which matches the concentration used by Zilberter and colleagues in their experiments.

Note also that lactate and pyruvate seem to be quite similar, and possibly interchangeable, in respect to their metabolism (Zilberter et al. 2010), effect on intracellular pH (Ruusuvuori et al. 2010), GDP generation (Holmgren et al. 2010; Ruusuvuori et al. 2010), modulation of synaptic function (Juge et al. 2010), and other aspects, as briefly reviewed in Holmgren et al. (2010). It further supports the assumption that the results of Holmgren et al. (2010) observed in pyruvate-containing ACSF are physiologically adequate, even although it is lactate, and not pyruvate, that is actually present in high concentrations in the extracellular fluid in vivo (Tyzio et al. 2011; Zilberter et al. 2010).

Can the effect of weak acids on GABA reversal potential be attributed to the specificity of immature brain metabolism? There are indications that neural cells, unlike glia, prefer lactate to glucose metabolically (Pellerin et al. 2007), and that energy sources other than glucose can be especially important at early developmental stages (Zilberter et al. 2010). However, when mitochondrial membrane potential was measured with a fluorescent probe in slices immersed in ACSF solutions of different compositions, it was found that enhancement of ACSF with weak acids did not change this potential relative to that recorded in a glucose-based solution. Weak acids also failed to support the mitochondrial membrane potential at a normal level in the absence of glucose (Ruusuvuori et al. 2010). As the mitochondrial membrane potential is a strong indicator of the aerobic cell metabolism efficiency, these results demonstrate that the effect of weak acids on GABA transmission does not correlate with changes in metabolism, and so cannot be attributed to them.

Can the effect of weak acids on GABA reversal potential be attributed to changes in the intracellular pH? If weak acids do not improve cellular metabolism, as it was initially suggested, how could they exert their effect on GABA reversal potential? The most plausible answer is that weak acids induce intracel-
lular acidification (Ruusuvuori et al. 2010), which in turn triggers a well-known cascade of homeostatic reactions (Fig. 1B). First, a drop in pH would shift equilibrium for the carbonic anhydrase (an enzyme catalyzing conversion of bicarbonate and protons into carbon dioxide and water and vice versa), decreasing intracellular bicarbonate ions concentration. Then, a decrease of bicarbonate concentration would activate SLC4 AE3 bicarbonate-chloride cotransporter, which is known to be closely integrated with the carbonic anhydrase complex (Casey et al. 2009), bringing bicarbonate ions inside the cell, and pumping chloride ions out, thus reducing GABA reversal potential (Glykys et al. 2009).

It is easy to see that although in the work of Mukhtarov et al. (2011) a reduction in intracellular pH induced by a substitution of bicarbonate-containing extracellular media with a HEPES-based solution did not eliminate GDPs, but on the contrary increased their amplitude, there is no contradiction between this result and the hypothesis above. In the absence of bicarbonate ions in the external solution, the SLC4 AE3 would not be able to compensate for the decrease of the bicarbonate concentration in the external solution, the SLC4 AE3 would activate carbonic anhydrase to transform carbon dioxide into bicarbonate ions in the external solution, the SLC4 AE3 would thus reducing intracellular pH (Fig. 1B).

Are GDPs a good indicator for the weak acids action onto GABA transmission? Unlike the direct measurements of GABA reversal potential, which can be tricky and require elaborate techniques, GDP events seen in the immature brain tissue are slow, large, and easy to detect, which makes them useful as a primary indicator of depolarizing action of GABA. It turned out to be, however, that there is more than one way for weak acids to downregulate GDPs generation. It was recently shown that acetooacetate, pyruvate, and BHB at 1–10 mM concentrations affect glutamatergic transmission, reducing the amount of glutamate in presynaptic vesicles through a direct modulation of vesicular glutamate transporters (Juge et al. 2010). This effect is codirectional with the effect of weak acids on GABA transmission, and as there is no easy way to differentiate between them, direct measurements of GDPs generation should always be preferred to measurements of GDP frequency when the effects of weak acids are studied.

Conclusion: should weak acids become a standard component of the ACSF for immature brain preparations? From the most recent publications, presented in this article, one can conclude that 4–5 mM concentrations of lactate or pyruvate are likely to be physiologically relevant, accurately reproducing actual concentrations of weak acids in the extracellular fluid in vivo. On the other hand, at these concentrations, both lactate and pyruvate induce noticeable changes in GABA and glutamatergic transmission in developing neural networks. It means that some changes in experimental protocols and related theoretical paradigms may still be necessary. Although higher concentration of chloride ions in immature neurons is a well-established fact, it is still not clear if generation of propagating depolarization waves (GDPs) is really necessary for network maturation. Hopefully, new microdialysis studies, as well as studies of knockout mice lacking different chloride transporters, would make this picture more clear and lead to a scientific consensus. Until then it is probably useful to enhance ACSF with weak acids in studies of immature neural networks, at least to ensure that the observed qualitative phenomena are not too dependent on external solution composition.

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