Journal Club

Editor's Note: These short, critical reviews of recent papers in the *Journal*, written exclusively by graduate students or postdoctoral fellows, are intended to summarize the important findings of the paper and provide additional insight and commentary. For more information on the format and purpose of the Journal Club, please see http://www.jneurosci.org/misc/ifa_features.shtml.

Reality of Inhibitory GABA in Neonatal Brain: Time to Rewrite the Textbooks?

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Review of Valeeva et al.

The issue of GABA action in the developing brain has been debated for several years. The accepted axiom has been that, in neonatal brain, GABA exerts a mainly excitatory effect on the network, gradually switching to inhibitory action in the course of brain development (Ben-Ari et al., 2007). This switch was thought to be underlain by the observed developmental change in expression ratio of two major Cl^- transporters, NKCC1 (which increases $[Cl^-]_i$) and KCC2 (which decreases $[Cl^-]_i$), leading to a change from higher $[Cl^-]_i$, and therefore to a more depolarized reversal potential for GABA receptor-mediated Cl^- currents (E_{GABA}), in immature mice to lower $[Cl^-]_i$ and hyperpolarized E_{GABA} in mature rodents (Ben-Ari et al., 2007).

The validity of the excitatory GABA hypothesis was questioned, however, because it was based mainly on *in vitro* slice electrophysiology, which is susceptible to experimental artifacts (for review, see Bregestovski and Bernard, 2012). Furthermore, other mechanisms regulate neuronal $[Cl^-]_i$ in addition to NKCC1 and KCC2, including bicarbonate-chloride exchangers (Romero et al., 2004), Cl^- channels (Duran et al., 2010), and ATP-dependent Cl^- pumps (Inoue et al., 1991; Inagaki et al., 2001).

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It was therefore important to elucidate the effects of GABA receptor activation in the intact developing brain. Valeeva et al. (2016) provided much needed data in this respect, using the same experimental technique *in vivo* and in acute brain slices to demonstrate the discrepancy between results obtained under the two conditions.

In postnatal day 3–9 (P3-P9) mice, optogenetic activation of channelrhodopsin-2 (ChR2) expressed in GABAergic interneurons reduced the frequency of EPSCs in cortical neurons, suggesting that GABA exerted an inhibitory effect on the network. These data confirmed the results of another recent study (Kirmse et al., 2015) in which different techniques were used to show that, in P3-P4 animals, the reversal potential of GABA-mediated currents (E_{GABA}) was close to the resting membrane potential (E_m) and that GABA application failed to increase neuronal activity, suggesting an overall inhibitory mode of action.

In contrast to the *in vivo* results, experiments performed on acute slices from P2-P8 animals conclusively showed GABA to increase the EPSC frequency, suggesting an excitatory effect; this differed from results obtained in slices from older P9-P15 animals where a reduction of EPSC frequency upon GABA activation suggested an inhibitory effect. These data confirmed the results from several classic slice studies on which the theory of developmental excitatory/inhibitory switch of GABA signaling was based (for review, see Ben-Ari et al., 2007).

The obvious question, then, is what explains this striking difference between the *in vitro* and *in vivo* results, especially because the experimental techniques used for both were nearly identical. The authors investigated potential artifacts by modifying slice experimental conditions. Because animals were anesthetized during *in vivo* recordings, it was important to rule out any effects of anesthesia itself. Indeed, the authors found that GABA remained excitatory in slices exposed to 10 mM urethane. The authors also modified the slicing procedure (using a choline-based solution), ACSF electrolyte composition (to more closely resemble that of *in vivo* environment), and temperature (to 36°C), but the excitatory GABA action persisted. The reason for excitatory GABA effects in immature brain slices thus remained unanswered. Nonetheless, several potential mechanisms were not addressed.

It is well known that acute brain slices are not always a reliable experimental model, and one must take care to extrapolate data, including those on mode of GABAergic effects, onto an *in vivo* situation (Zilberter et al., 2010). There have been a number of studies showing that GABAergic signaling could be affected by conditions and alterations in acute brain slices, those not accounted for by the authors.

Because GABA's inhibitory effects in the adult brain are known to be reversed to excitatory by pathological conditions such as epilepsy and traumatic brain injury, GABA actions in neonatal brain slices might be altered by acute neuronal damage suffered during the slicing procedure (Dzhala et al., 2012). Using a Cl^- optical sensor in acute neonatal slices, Dzhala et al. (2012) showed

that increased neuronal $[Cl^-]_i$ (resulting in depolarized E_{GABA}) was correlated with proximity to the slice surface and with neuronal damage, and that $GABA_A$ receptor activation by bath-applied isoguvacine was excitatory to the network at depths of 80–100 μm. In contrast, in intact (*in toto*) neonatal hippocampal preparations, calculated E_{Cl} in neurons proximal to the surface was close to or below the assumed E_m, and GABA activation by isoguvacine exerted inhibitory effects on spontaneous network activity, basically showing the same discrepancy in E_{GABA} measurements and GABA effects in intact preparations versus brain slices. These results were also consistent with reports from an earlier study showing E_{GABA} in intact immature hippocampus to be close to the neuronal E_m (Wong et al., 2005).

Thus, acute brain damage might explain the results of Valeeva et al. (2016), but because the study's main readout of GABA effects was the frequency of EPSCs, which are evoked by hundreds of presynaptic glutamatergic cells converging onto a single recorded neuron, one can assume that a large portion of presynaptic cells were located deeper into the slice and therefore relatively undamaged. In addition, a modified slicing procedure that would presumably increase the "health" of the slice did not change the GABA excitatory mode of action. Thus, the fact that GABA activation still increased total network activity suggests that the main artifact is not attributable to neuronal damage.

Another potential mechanism behind artifactual GABA action measurements in immature slices is insufficient energy metabolism substrates provided by ACSF perfusion. Glucose utilization is limited in the developing rodent brain, at only 20% of the adult levels (Vannucci and Vannucci, 2000) and other energy substrates, such as lactate or ketone bodies (Nehlig, 2004), are used instead. It has been shown that, in immature slices, unlike in mature brains, lactate oxidation in the tricarboxylic acid cycle is 10 times higher than that of glucose (Medina, 1985). Enzymatic systems involved in glucose utilization follow a developmental profile (Prins, 2008), starting to mature right about the time when Valeeva et al. (2016) reported a switch from excitatory to inhibitory GABA effects in slices. This could potentially explain their data, given that HCO_3^- Cl^- exchangers depend on intracellular ATP (Romero et al., 2004; Chen et al., 2008), as do Cl^- ATPases (Inoue et al., 1991; Inagaki et al., 2001). Indeed, other studies reported that supplementing ACSF with alternative energy substrates, such as β-hydroxybutyrate (BHB), pyruvate, or lactate, improved the metabolic state of neurons (Ivanov and Zilberter, 2011) and significantly hyperpolarized E_{GABA}, switching the GABA action in immature slices from excitatory to inhibitory; this effect depended on the action of HCO_3^- Cl^- transporters (Holmgren et al., 2010). The effect of lactate on reversing GABA action in neonatal slices was confirmed (Ruusuvuori et al., 2010), although those authors contributed it to acidification, a theory later disproven (Mukhtarov et al., 2011). It is therefore unfortunate that Valeeva et al. (2016) did not consider adding alternative energy substrates in modifying ACSF composition to more closely resemble that of *in vivo* conditions.

Even if Valeeva et al. (2016) did add lactate or BHB to their ACSF, this may not have been sufficient to reverse the GABA effects, if there was insufficient oxygenation (Hájos et al., 2009). The authors used an ACSF perfusion rate of 2–4 ml/min, but it has been shown that, at these rates, most neurons in neonatal slices are under acute hypoxia (Ivanov et al., 2011), with neurons unable to efficiently use any fuel for mitochondrial respiration and production of ATP needed to sustain physiological $[Cl]_i$.

Despite the failure to identify the cause for differences in GABA effects *in vivo* and *in vitro*, the study by Valeeva et al. (2016) is significant in several respects. Most important, the study conclusively shows and confirms the general inhibitory mode of GABAergic action in neonatal brain, helping put to rest years of controversy and suggesting the need to rewrite the textbooks. Quoting another recent study on *in vivo* inhibitory GABA in neonatal brain (Kirmse et al., 2015), ". . . an absence of GABA-mediated excitation . . . could have major implications for a central hypothesis of developmental neurobiology." The second important finding of the study is that results obtained *in vitro* and *in vivo* differ even when the same experimental technique is used to measure GABA effects. The recorded mode of GABA action in acute brain slices was directly opposite to that observed in live animals of the same age. Future work should investigate whether energy metabolism correction (such as addition of lactate or BHB to ACSF together with sufficient oxygenation), which has been reported to reverse GABA action in neonatal brain slices, would lead to similar results using this technique. These results perfectly illustrate that, although acute brain slices are extremely useful for elucidating brain function, they are severely limited in several aspects due to the nature of the preparation, and great care should be taken in extrapolating and interpreting data obtained from such experiments (Zilberter et al., 2010; Kann, 2011).

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