



## Carts, Horses, and Push-Pull Regulation of EGABA in Neonatal Seizures

### Neuronal Chloride Accumulation and Excitatory GABA Underlie Aggravation of Neonatal Epileptiform Activities by Phenobarbital.

Nardou R, Yamamoto S, Chazal G, Bhar A, Ferrand N, Dulac O, Ben-Ari Y, Khalilov I. *Brain*. 2011;134(Pt 4):987–1002.

Phenobarbital produces its anti-epileptic actions by increasing the inhibitory drive of gamma-aminobutyric acid. However, following recurrent seizures, g-aminobutyric acid excites neurons because of a persistent increase of chloride raising the important issue of whether phenobarbital could aggravate persistent seizures. Here we compared the actions of phenobarbital on initial and established ictal-like events in an in vitro model of mirror focus. Using the in vitro three-compartment chamber preparation with the two hippocampi and their commissural fibres placed in three different chambers, kainate was applied to one hippocampus and phenobarbital contralaterally, either after one ictal-like event or after many recurrent ictal-like events that produce an epileptogenic mirror focus. Field, perforated patch and single-channel recordings were used to determine the effects of g-aminobutyric acid and their modulation by phenobarbital, and alterations of the chloride cotransporters were investigated using sodium–potassium–chloride cotransporter 1 and potassium chloride cotransporter 2 antagonists, potassium chloride cotransporter 2 immunocytochemistry and sodium–potassium–chloride cotransporter 1 knockouts. Phenobarbital reduced initial ictal-like events and prevented the formation of a mirror focus when applied from the start. In contrast, phenobarbital aggravated epileptiform activities when applied after many ictal-like events by enhancing the excitatory actions of g-aminobutyric acid due to increased chloride. The accumulation of chloride and the excitatory actions of g-aminobutyric acid in mirror foci neurons are mediated by the sodium–potassium–chloride cotransporter 1 chloride importer and by downregulation and internalization of the chloride-exporter potassium-chloride cotransporter 2. Finally, concomitant applications of the sodium–potassium–chloride cotransporter 1 antagonist bumetanide and phenobarbital decreased excitatory actions of g-aminobutyric acid and prevented its paradoxical actions on mirror focus. Therefore, the history of seizures prior to phenobarbital applications determines its effects and rapid treatment of severe potentially epileptogenic-neonatal seizures is recommended to prevent secondary epileptogenesis associated with potassium chloride cotransporter 2 downregulation and acquisition of the excitatory g-aminobutyric acid phenotype.

### Commentary

In this interesting study, Ben-Ari and colleagues continue their study of synaptic GABA signaling in the developing brain. This group pioneered the idea that excitatory actions of GABA in the immature brain could contribute to physiological synchronization of neuronal activity in normal brain slices (1). This study examines whether excitatory actions of GABA contribute to anticonvulsant failure in the treatment of neonatal seizures.

GABAA receptors operate an anion-permeable channel in the cytoplasmic membrane that is permeable to chloride and bicarbonate ions. Because chloride is five times more permeable and the extracellular concentration is four times the concentration of bicarbonate (2), the concentration of chloride inside the neuron is the principal determinant of

the direction of ion flow through open GABAA channels: inward (inhibitory) or outward (excitatory). The concentration of chloride is considered to be regulated by two transporters: a sodium-potassium-chloride transporter called NKCC1 and a potassium chloride cotransporter, KCC2 (3). NKCC1 is expressed early in development, and at typical ion concentrations is a net importer of chloride. KCC2 is expressed later in development, and at typical ion concentrations is a net exporter of chloride. Because high intracellular chloride concentrations can reverse the flow of ions through the GABA receptor, the chloride importer NKCC1 can reduce the efficacy of GABA-mediated inhibition. This effect may be critical in neonatal seizures, which respond poorly to anticonvulsants that enhance the effects of GABA, such as barbiturates and benzodiazepines (4, 5). Bumetanide, a lipid-soluble inhibitor of NKCC1, which has been safely used as a diuretic in neonates for decades, is currently being tested as an adjunct to barbiturate treatment of neonatal seizures in clinical trials in the United States (6) and Europe (7).

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Did these clinical trials put the cart before the horse? The idea that high intracellular chloride explains failure of GABAergic inhibition and anticonvulsant therapy in neonatal seizures is attractive. But how strong is the evidence that NKCC1 is the culprit (8, 9)? Ben-Ari and colleagues (1) made clever use of a preparation they developed several years ago in which both hippocampi and their septal connections are dissected intact from a perinatal rodent brain. Perfusing each side separately permits exposure of one hippocampus to convulsant and the other hippocampus to epileptiform input via the septal connections, but importantly, no exposure to the chemoconvulsant. The hippocampus that is not exposed to the chemoconvulsant nevertheless develops epileptiform activity as a consequence of driving from the exposed hippocampus, and after several seizures, the unexposed hippocampus develops spontaneous seizure activity. Although the Marseilles group prefers the term “mirror focus” to describe the unexposed hippocampus, this term is less popular elsewhere because of the implied chronicity characteristic of human mirror foci.

Ben-Ari and colleagues (1) confirmed that GABAergic anticonvulsants are inhibitory in control conditions; and after seizure activity was induced with kainate in one hippocampus, seizure activity in the unexposed hippocampus was inhibited by phenobarbital. A key finding was that the efficacy of phenobarbital was time limited: the drug had to be applied before the first one or two seizures to be effective. After the unexposed hippocampus had experienced several seizures, phenobarbital failed to repress seizures and exacerbated epileptiform activity in slices prepared from these hippocampi. The authors also provide important confirmation of the findings on which the clinical trials are based (4, 5): after several seizures, the anticonvulsant effect of phenobarbital could be restored by antagonizing the chloride-importing cotransporter NKCC1 with bumetanide. The utility of bumetanide in neonatal seizures has now been confirmed by this study and several others (10–13), and its increasing efficacy with the number of preceding seizures has now been established by Nardou et al. of this report and by two additional laboratories (14, 15), so the weight of current evidence suggests that the cart is behind the horse.

Although the current study found that bumetanide had a substantial anticonvulsant effect in combination with a GABAergic anticonvulsant, neither bumetanide nor knockout of NKCC1 prevented the development of epileptiform activity in the hippocampus that was exposed to epileptiform activity but no convulsant. This finding is congruent with acute kindling experiments in adult animals (16), in which neurons also do not express significant NKCC1 (4). In the presence of repeated epileptiform stimulation, there are unfortunately many other mechanisms of GABAergic inhibitory failure besides NKCC1, including endocytosis of GABA receptors (17, 18) and shift of EGABA due to preferential maintenance of bicarbonate versus chloride gradients (19).

Digging deeper into the mechanism of bumetanide's enhancement of the anticonvulsant effect of barbiturate, the authors report several more puzzling findings. They discovered that block of NKCC1 in control preparations reduced chloride efflux through the GABA receptor, as would be expected if NKCC1 were accumulating chloride. The effect of bumetanide

on GABA-gated chloride efflux was much larger in neurons that experienced seizure activity. This is consistent with the anticonvulsant effect mentioned above, and with prior reports of activity-dependent chloride accumulation in perinatal brain tissue (15, 20), but it does not clarify the mechanism.

To address the mechanism of activity-dependent chloride accumulation in developing neurons, the authors measured chloride transport rates in naïve neurons and in those that had experienced seizure activity, concluding that seizures reduced the chloride transport rates. Performing similar experiments, we found that a series of action potentials did not change the transport kinetics but did change the steady-state resting chloride concentration (20). We interpreted these data as evidence for a change in the steady-state concentrations of the cotransported potassium and sodium ions, because the concentrations of these cations determine the equilibrium concentration of chloride; and further, blocking sodium-potassium ATPase prevented the increase in cytoplasmic chloride. The current study also found activity-dependent increases in the steady-state driving forces for chloride but neglected this change when fitting the data (all chloride currents were normalized prior to fitting exponential decay curves). Because transport rates are proportional to the chloride load, normalization introduces a distortion when the chloride driving forces and consequent chloride loading differ in control and experimental neurons. Further, although the GABA current amplitude is related to the intracellular chloride concentration, the relationship is not linear; because chloride is the transported species, the mono-exponential decay expected of a first-order transport process applies to the chloride concentration rather than current amplitude. In light of these issues, the conclusion that the chloride transport rate is changed by seizures may be premature.

A second puzzling finding is that while block of NKCC1 reduced the driving force for GABA-gated chloride efflux as expected, in control neurons, the driving force was increased to twice the control value by blocking both NKCC1, the chloride importer, and KCC2, the chloride exporter. This finding is puzzling because with both the major transporters blocked, chloride should be passively distributed with minimal driving force for efflux or influx, rather than with maximal driving force, suggesting remarkable chloride accumulation. These experiments were performed using a laborious dual-patch technique that uses the reversal of cell-attached channel currents activated by NMDA and GABA in the two pipettes to determine the driving force for GABA. This should be a very noninvasive technique to measure the driving force for chloride—there is no rupture of the neuronal membrane, so the intracellular milieu is not altered. However, this technique relies on continued GABA-mediated currents under the patch pipette, and it might be that these currents led to inadvertent alterations in the local chloride concentration. The only other explanation is that a large, ongoing influx of chloride from some source other than NKCC1 (which was also blocked) was being balanced by outward KCC2 transport, so that block of KCC2 resulted in increased cytoplasmic chloride from the unidentified source. For example, ongoing chloride flux from phasic and tonic GABAA receptor activation could potentially comprise such a chloride source, but this seems unlikely in light of the transport kinet-



ics reported here and elsewhere—the charge transferred by such GABA currents would need to be unrealistically large in order to sufficiently load the KCC2 transporter. Further, the very large driving force for chloride efflux induced by the KCC2 block should cause GABA<sub>A</sub>-receptor activation to become exceptionally excitatory—yet there are no reports of seizures when the KCC2 is blocked in mature or developing slices, and the KCC2 antagonist furosemide has anticonvulsant effects in experimental and human epilepsy (21). This issue can be readily resolved by repeating the experiment in the presence of a GABA antagonist, and using a fluorometric chloride indicator to read out the cytoplasmic chloride.

The authors also evaluated KCC2 by immunohistochemistry and electron microscopy and argue that after seizures, KCC2 is less membrane bound than in control conditions. Removal of KCC2 from the membrane is dependent on both activity and phosphorylation (22), and altered KCC2 membrane trafficking has been observed in adult preparations as a consequence of interictal activity (23). The authors argue that seizures induce a reduction in membranous KCC2, allowing chloride accumulation, reduced efficacy of GABA<sub>A</sub> receptor-mediated inhibition, and reduced efficacy of GABAergic anticonvulsants. The problems with this idea are that others have found increases in KCC2 activity under similar conditions (24, 25); the kinetic analysis does not support a reduction in transport; the increase in driving force requires a large, unidentified chloride source; and finally, the KCC2 transport rate required to negate chloride influx from NKCC1 and the unidentified source would rapidly run down the transmembrane potassium gradient that powers KCC2, requiring an equally large source of ATP to restore the potassium gradient via sodium–potassium ATPase. Returning to the cart and horse analogy—KCC2 pumping chloride out of the neuron, and NKCC1 and another source bringing it in, this amounts to hitching one horse to the back of the cart and one to the front, and asking them to pull against each other. While this might allow for very precise location of the cart, it is clearly an enormous waste of energy, and the same is true for simultaneous, oppositely directed chloride transport. Opposing transport rates in the range of micromoles per liter per minute might be OK, because the molar ATP consumed would be trivial. But the chloride fluxes described here are many millimoles per liter per minute, and even higher transport rates have been measured in neurons at this stage of development (20). While we do not yet have final answers as to how neurons regulate cytoplasmic chloride and EGABA in health and disease, an important aspect of any potential solution should be minimization of push–pull chloride transport. However attractive this may be from the point of view of regulation, think of the poor horses.

by Kevin Staley, MD

## References

- Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL. Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J Physiol*. 1989;416:303–325.
- Bormann J, Hamill OP, Sakmann B. Mechanism of anion permeation through channels gated by glycine and gamma-aminobutyric acid in mouse cultured spinal neurones. *J Physiol*. 1987;385:243–286.
- Blaesse P, Airaksinen MS, Rivera C, Kaila K. Cation-chloride cotransporters and neuronal function. *Neuron*. 2009;61:820–838.
- Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, Delpire EJ, Jensen FE, Staley KJ. NKCC1 transporter facilitates seizures in the developing brain. *Nat Med*. 2005;11:1205–1213.
- Dzhala V, Brumback A, Staley KJ. Bumetanide enhances phenobarbital efficacy in a neonatal seizure model. *Ann Neurol*. 2008;63:222–235.
- Frances J, Citizens United for Research in Epilepsy, Charles H. Hood Foundation, National Institute of Neurological Disorders and stroke. Pilot study of bumetanide for newborn seizures. Clinicaltrials.gov Web site. Clinicaltrials.gov identifier #NCT00830531. Accessed Month day, year.
- NEMO. The NEMO-Europe Project. NEMO Web site. <http://www.nemo-europe.com>. Accessed Month day, year.
- Vanhatalo S, Hellström-Westas L, De Vries LS. Bumetanide for neonatal seizures: Based on evidence or enthusiasm? *Epilepsia*. 2009;50:1292–1293.
- Chabwine JN, Vanden Eijnden S. A claim for caution in the use of promising bumetanide to treat neonatal seizures. *J Child Neurol*. 2011;26:657–658.
- Mazarati A, Shin D, Sankar R. Bumetanide inhibits rapid kindling in neonatal rats. *Epilepsia*. 2009;50:2117–2122.
- Edwards DA, Shah HP, Cao W, Gravenstein N, Seubert CN, Martynuk AE. Bumetanide alleviates epileptogenic and neurotoxic effects of sevoflurane in neonatal rat brain. *Anesthesiology*. 2010;112:567–575.
- Wahab A, Albus K, Heinemann U. Age- and region-specific effects of anticonvulsants and bumetanide on 4-aminopyridine-induced seizure-like events in immature rat hippocampal-entorhinal cortex slices. *Epilepsia*. 2011;52:94–103.
- Nardou R, Ben-Ari Y, Khalilov I. Bumetanide, an NKCC1 antagonist, does not prevent formation of epileptogenic focus but blocks epileptic focus seizures in immature rat hippocampus. *J Neurophysiol*. 2009;101:2878–2888.
- Kilb W, Sinning A, Luhmann HJ. Model-specific effects of bumetanide on epileptiform activity in the in-vitro intact hippocampus of the newborn mouse. *Neuropharmacology*. 2007;53:524–533.
- Dzhala V, Kuchibhotla KV, Glykys J, Kahle KT, Swiercz W, Feng G, Kuner T, Augustine G, Bacskai BJ, Staley KJ. Progressive NKCC1-dependent neuronal chloride accumulation during neonatal seizures. *J Neurosci*. 2010;30:11745–11761.
- Lothman EW, Hatlelid JM, Zorumski CF, Conry JA, Moon PF, Perlin JB. Kindling with rapidly recurring hippocampal seizures. *Brain Res*. 1985;360:83–91.
- Blair RE, Sombati S, Lawrence DC, McCay BD, DeLorenzo RJ. Epileptogenesis causes acute and chronic increases in GABA<sub>A</sub> receptor endocytosis that contributes to the induction and maintenance of seizures in the hippocampal culture model of acquired epilepsy. *J Pharmacol Exp Ther*. 2004 Sep;310(3):871–80.
- Goodkin HP, Sun C, Yeh JL, Mangan PS, Kapur J. GABA(A) receptor internalization during seizures. *Epilepsia*. 2007;48(suppl):109–113.
- Staley KJ, Soldo B, Proctor W. Ionic mechanisms of neuronal excitation by inhibitory GABA<sub>A</sub> receptors. *Science*. 1995;269:977–981.
- Brumback A, Staley KJ. Thermodynamic regulation of NKCC1-mediated Cl<sup>-</sup> cotransport underlies plasticity of GABA<sub>A</sub> signaling in neonatal neurons. *J Neurosci*. 2008;28:1301–1312.



21. Haglund MM, Hochman DW. Furosemide and mannitol suppression of epileptic activity in the human brain. *J Neurophysiol.* 2005;94:907–918.
22. Lee HH, Jurd R, Moss SJ. Tyrosine phosphorylation regulates the membrane trafficking of the potassium chloride co-transporter KCC2. *Mol Cell Neurosci.* 2010;45:173–179.
23. Rivera C, Voipio J, Thomas-Crusells J, Li H, Emri Z, Sipilä S, Payne JA, Minichiello L, Saarma M, Kaila K. Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter KCC2. *J Neurosci.* 2004;24:4683–4691.
24. Galanopoulou AS. GABA(A) receptors in normal development and seizures: Friends or foes? *Curr Neuropharmacol.* 2008;6:1–20.
25. Khirug S, Ahmad F, Puskarjov M, Afzalov R, Kaila K, Blaesse P. A single seizure episode leads to rapid functional activation of KCC2 in the neonatal rat hippocampus. *J Neurosci.* 2010;30:12028–12035.



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