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Clarifications regarding bumetanide for neonatal seizures

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Neonatal seizures are frequently resistant to anticonvulsant therapy[1]. My colleagues and I have pursued the idea that neuronal chloride accumulation after brain injury produces a positive shift in the reversal potential for GABA-gated chloride currents that compromises GABAergic inhibition[2]. After injury, inhibiting the sodium-potassium-chloride cotransporter NKCC1 reduces neuronal chloride accumulation[3], which shifts the GABA reversal potential to more physiological values, and improves the response of neonatal seizures to anticonvulsants such as phenobarbital that increase the mean open time of $GABA_A$ channels[4]. These ideas were challenged in a recent study[5] and subsequent editorial[6] by the study authors. Here I respond to those challenges.

The core challenge is that bumetanide was not effective in a new experimental model of neonatal seizures[5]. However, several aspects of this neonatal seizure model[5,7,8,9] may reduce its accuracy as a predictor of anticonvulsant efficacy for human neonatal seizures. The model is closely based on the hypercarbia-withdrawal model of acute seizures developed by Dixon Woodbury and colleagues in the 1950s[10,11,12]. In these models, seizures are triggered by the termination of exposure to hypercarbia, with or without accompanying hypoxia. The extension of this model to predict anticonvulsant efficacy for neonatal seizures raises several concerns:

1. The hypercarbia-withdrawal model does not predict the efficacy of anticonvulsants in human seizures. For example, Woodbury et al. found that seizures are *worsened* by therapeutic doses of phenytoin[12], but the opposite occurs in human neonatal seizures[13].
2. The primacy of hypercarbia withdrawal (vs hypoxic ischemic brain injury) as a driver of ictogenesis in the hypercarbia withdrawal model is underscored by the unique responsiveness of these seizures to agents that slow the response to

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hypercarbia withdrawal[14]. Slowing the re-equilibration of CO_2 with the carbonic anhydrase inhibitor acetazolamide blocked seizures both in Woodbury's original reports[10, 11] and in the hypercarbia withdrawal model of neonatal seizures[8].

3. Hypoxia and ischemia are not necessary to induce seizures by hypercarbia withdrawal in perinatal animals[15], weakening the rationale for the withdrawal of hypercarbia as a model of human seizures following a perinatal hypoxic-ischemic insult.

4. Hypercarbia can occur in human perinatal asphyxia, but the levels of CO_2 in the umbilical artery[16,17] almost never reach the levels required to induce hypercarbia withdrawal seizures[5, 7 , 9, 11, 11, 12]. Importantly, the umbilical artery PCO_2 does not predict neonatal seizures in humans[18,19].

5. The difference in mechanisms underlying hypercarbia-withdrawal vs human neonatal seizures is highlighted by the large differences in the time course of seizures. Seizures begin within 30 seconds of CO_2 withdrawal, and terminate within a few minutes of the reduction in inspired CO_2[9, 10, 11], similar to the time course of absence seizures triggered by hyperventilation-induced reductions in systemic CO_2[20,21]. But seizures in asphyxiated human newborns begin hours[22,23], not seconds, after delivery, and for this reason neonatal resuscitation guidelines do not mention seizure management[24,25]. Neonatal seizures associated with hypoxic ischemic brain injury continue for hours to days, not minutes, after establishment of stable ventilation[22, 23].

For these reasons, a lack of effect of bumetanide in the perinatal hypercarbia-withdrawal model does not significantly challenge the demonstrated efficacy of bumetanide in several models of experimental neonatal seizures[2,26,27,28,29,30,31,32], neonatal brain injury[33], and the robust efficacy signal produced in the blinded, randomized, controlled trial treating seizures in human newborns[34].

In the subsequent editorial, the theoretical basis for the efficacy of bumetanide for neonatal seizures was questioned based on the levels of the membranous cation-chloride transporters present in the neonatal human brain. In immunohistochemical and Western blot studies of human brain, we found higher neonatal levels of neuronal NKCC1 and lower levels of KCC2[2], the neuron-specific potassium-chloride transporter that canonically exports chloride to maintain low cytoplasmic chloride concentrations and an inhibitory, hyperpolarizing $GABA_A$ reversal potential. The editorial argued that other studies found higher levels of KCC2 in the human neonatal brain as evidence that the $GABA_A$ reversal potential was already sufficiently hyperpolarizing in human neonates, such that inhibition of NKCC1 by bumetanide would not be an effective anticonvulsant therapy. I have the following concerns with this idea.

1. Of the 4 studies cited in the editorial to compare neonatal to mature KCC2 levels, one did not study KCC2 levels in the mature brain[35]. 2 of the studies measured mRNA rather than the level of KCC2 protein[35,36]. Of the two immunohistochemical studies, one states "the expression of KCC2 continues to

increase throughout the third trimester and postpartum period"[37], which supports our findings[2].

2. The relative activity of KCC2 is a clearly established determinant of seizure termination[38], and mechanisms to fractionally enhance KCC2 activity are being pursued as anticonvulsant strategy[39]. We showed a 3 – 4 fold increase in human cortical KCC2 protein from term age to adult using Western blots[2]. The mRNA studies cited in the editorial also show an increase in KCC2 message from term newborn to adult brain, in the range of 50 – 100%[36,40]. All of these reported changes in KCC2 expression are relevant to neonatal seizure control.

3. At the ages considered equivalent to term birth in humans[41,42], a wealth of experimental data, with key contributions from the authors of the editorial[43], demonstrate that KCC2 expression is low in rodents (reviewed in 44). The editorial authors have also shown that excitatory hippocampal activity in rodents at these ages is suppressed by bumetanide in vivo[45].

4. Our understanding of neuronal chloride homeostasis has evolved beyond the idea that KCC2 expression dictates whether GABA transmission is hyperpolarizing. We have demonstrated that the species of expressed cation-chloride transporter (i.e. NKCC1 vs KCC2) does not determine the neuronal cytoplasmic chloride concentration or the polarity of $GABA_A$ responses[46,47]. While this finding was initially controversial[48,49,50], the key findings (e.g. neuronal chloride concentrations do not collapse to a passive distribution after block of chloride transporters) have since been replicated by the editorial authors and colleagues (cf. Figure 1B,C of reference 46 and Figure 6D of reference 51); the key predictions (e.g. the redistribution of other charged cytoplasmic molecules have a larger immediate effect on chloride concentrations than block of either chloride transporter) have recently been demonstrated[52]; and the implications for the anticonvulsant effects of bumetanide are discussed in detail in a review[53] and in the trial paper[34].

Thus the concerns regarding KCC2 expression in the neonatal brain do not address the basis for the efficacy of bumetanide.

The editorial raises a valid question regarding the distribution of bumetanide in the brain. Experimental studies perfuse bumetanide directly onto neurons, whereas in vivo bumetanide must first cross the blood-brain barrier (BBB). However, in experimental studies, chloride salts are also applied directly to neurons. Whereas in vivo, chloride salts must first cross the blood brain barrier after brain injury before entering neurons to cause cytotoxic edema and shift the $GABA_A$ reversal potential[53,54]. NKCC1 is an important component of the chloride pathway from blood to brain[55,56]. Thus after brain injury and seizures, systemic bumetanide limits not only seizures in neonatal animals[2, 26, 30, 32] but also cerebral edema in mature animals[57,54,58]. Highlighting the potential role of NKCC1 at the BBB, the only models in which bumetanide is not effective have either no perfusion of the injured brain tissue[59], or directly injure the BBB itself[60]. Thus while the action of bumetanide is to lower neuronal cytoplasmic chloride, the locus at which this effect occurs may be either at the neuronal membrane or the blood brain barrier.

The editorial also raises the concern that bumetanide may be ototoxic. It is important to recognize that most of the patients treated for neonatal seizures have hypoxic-ischemic encephalopathy (HIE)[34, 63], and there is a 10% incidence of hearing loss in neonatal HIE treated with hypothermia[61]. Experimental studies in non-asphyxiated animals have demonstrated no evidence of bumetanide otoxtoxicity at a dose of 50 mg / kg[62,63], which is 150–500 times the dose used in the human neonatal seizure trials[34,64]. Although bumetanide has not been shown to be ototoxic experimentally, bumetanide and other loop diuretics increase aminoglycoside ototoxicity[61, 62, 65]. Accordingly, *none of the patients in our study who suffered hearing loss were treated with bumetanide alone* (Table). 2 patients with hearing loss were treated with aminoglycosides and bumetanide, and one patient with hearing loss was enrolled but not treated (did not meet criteria regarding seizure activity for randomization). It is important to clarify that while the editorial correctly states that a neonate who did not receive gentamicin in our study suffered hearing loss, the editorial does not indicate that the neonate in question *also did not receive bumetanide* (Table). As stated in our trial paper, the numbers are too low in either our trial[34] or the preceding NEMO trial[63] (or both trials combined) to reach statistical significance. Thus, the blinded, randomized trial provides no evidence that bumetanide in the absence of aminoglycosides is ototoxic. However, future studies of bumetanide should be designed to avoid co-administration of aminoglycoside antibiotics.

Finally, the editorial questions why we did not provide the fractional reduction in neonatal seizures for phenobarbital +/− bumetanide. The reduction in seizures for each group is plotted vs the overall seizure burden in Figures 5 and 6[34]. The fractional reduction in seizures is then the slopes of these plots for phenobarbital and phenobarbital + bumetanide, which are provided. The large difference in these slopes (Figure 5) and the consistent effect of bumetanide dose on the slope shown in Figure 6 provide strong antiseizure efficacy signals for the addition of bumetanide vs. phenobarbital alone.

The next step is a randomized, controlled, multicenter Phase II-III trial of bumetanide for neonatal seizures that do not respond to phenobarbital, excluding neonates treated with aminoglycosides.

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Table:

hearing loss vs bumetanide and bumetanide + aminoglycoside therapy for the 26 surviving treated neonates in the Boston neonatal seizure trial[3].

	Hearing loss	No hearing loss
bumetanide	0	12
Aminoglycoside + bumetanide	2	12