The bumetanide-sensitive Na-K-2Cl cotransporter NKCC1 as a potential target of a novel mechanism-based treatment strategy for neonatal seizures

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Seizures that occur during the neonatal period do so with a greater frequency than at any other age, have profound consequences for cognitive and motor development, and are difficult to treat with the existing series of antiepileptic drugs. During development, γ-aminobutyric acid (GABA)ergic neurotransmission undergoes a switch from excitatory to inhibitory due to a reversal of neuronal chloride (Cl−) gradients. The intracellular level of chloride ([Cl−]i) in immature neonatal neurons, compared with mature adult neurons, is about 20-40 mM higher due to robust activity of the chloride-importing Na-K-2Cl cotransporter NKCC1, such that the binding of GABA to ligand-gated GABA_A receptors triggers Cl− efflux and depolarizing excitation. In adults, NKCC1 expression decreases and the expression of the genetically related chloride-extruding K-Cl cotransporter KCC2 increases, lowering [Cl−]i to a level such that activation of GABA_A receptors triggers Cl− influx and inhibitory hyperpolarization. The excitatory action of GABA in neonates, while playing an important role in neuronal development and synaptogenesis, accounts for the decreased seizure threshold, increased seizure propensity, and poor efficacy of GABAergic anticonvulsants in this age group. Bumetanide, a furosemide-related diuretic already used to treat volume overload in neonates, is a specific inhibitor of NKCC1 at low doses, can switch the GABA equilibrium potential of immature neurons from depolarizing to hyperpolarizing, and has recently been shown to inhibit epileptic activity in vitro and in vivo in animal models of neonatal seizures. The fundamental role of NKCC1 in establishing excitatory GABAergic neurotransmission in the neonate makes it a tempting target of a novel mechanism-based anticonvulsant strategy that could utilize the well-known pharmacology of bumetanide to help treat neonatal seizures. (DOI: 10.3171/FOC/2008/25/9/E22)

KEY WORDS • cation-chloride cotransporter • chloride transport • γ-aminobutyric acid • KCC2 • NKCC1 • neonatal seizures

Abbreviations used in this paper: [Cl−]i = intracellular concentration of chloride; CNS = central nervous system; ECl = chloride equilibrium potential; EEG = electroencephalogram; EGABA = GABA equilibrium potential; GABA = γ-aminobutyric acid; K_i = inhibition constant; TLE = temporal lobe epilepsy.
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Neonatal seizures, or epileptic episodes suffered by infants in the first 28 days of life, occur in 1–2% of patients in neonatal intensive care units, and are the most common manifestation of an acute neurological disorder in newborn infants.75 Most commonly caused by hypoxic ischemic encephalopathy, hemorrhage, or cerebral infarction, the presence of neonatal seizures often portends severe neurological dysfunction later in life, with high rates of adult epilepsy and long-term cognitive and motor deficits in survivors.13,75 In animal models, neonatal seizures have been shown to be injurious to the development of the brain, inducing synaptic reorganization, altering synaptic plasticity, and priming cortical neurons to increased damage from seizures sustained later in life.57 Thus, the prompt diagnosis and successful treatment of neonatal seizures is important for improving long-term neurological outcome.

Whereas seizure activity in adults is usually clinically obvious and the EEG reflects coordinated seizure activity, diagnosing seizures in neonates is difficult because seizures are often behaviorally subtle and the EEG typically demonstrates a multifocal process.86 Nonetheless, seizures in neonates are currently diagnosed using electroencephalography, with a discharge duration of 10 seconds required to diagnose an electrographic seizure (compared with 3 seconds in older age groups). However, because EEGs are not immediately available in many neonatal intensive care units, the initial diagnosis and treatment of seizures are often based on clinical assessment alone, and EEGs are obtained after the administration of antiepileptic drugs.61,65 Unfortunately, it is an all-too-common occurrence for electrographic seizures to persist in encephalopathic neonates despite anticonvulsant drugs at “therapeutic” levels.

Conventional antiepileptic drugs have limited utility in treating neonatal seizures.6,28,41 Barbiturates and benzodiazepines, which are GABA<sub>A</sub> receptor agonists that are efficacious for treating adult seizures, are currently among the first-line drugs for neonatal seizures; however, they are often ineffective, and have been shown to actually potentiate seizure activity in the immature brain.19,67 Phenytoin has been used with a similar amount of success.6,50 Barbiturates and benzodiazepines have also been known to produce a phenomenon termed “electroclinical dissociation” in neonates, whereby the overt clinical manifestations of seizures (convulsions) are inhibited, but electroencephalography-documented cortical seizure activity is either unaffected or exacerbated.16,62 This insidious effect of the GABA agonists has the potential for great harm, because it provides physicians with a false sense of security that seizures are under control, while cortical seizure activity—and its associated detrimental effects—continues.

There have been few prospective studies or randomized controlled trials of the antiepileptic drugs that are currently used to treat neonatal seizures.6,12,60 To date, the only randomized trial of antiepileptic drugs for the treatment of neonatal seizures compared the current first-line drugs (phenobarbital and phenytoin).50 In this study, the majority of neonates had asphyxia, infarction, or hemorrhage as the origin of their seizures. Complete control of electrographic seizures was achieved with either drug in only ~25% of neonates whose seizure frequency was increasing. Seizure control was achieved in another 15% of newborns when both agents were used concurrently. Preliminary studies of antiepileptic drugs other than phenobarbital and phenytoin have shown only modest efficacy in smaller cohorts of neonates, although sufficiently powered randomized trials are needed to conclusively demonstrate whether any of these drugs are truly effective.7,10,32,46,64 The lack of evidence-based treatment recommendations, coupled with the paucity of data regarding the underlying pathophysiology of neonatal seizures, has made their current management far from optimal.

The Strength and Polarity of GABAergic Neurotransmission Are Determined by the Intracellular Concentration of Chloride, Which Is Established by NKCC1 and KCC2

Recent research has provided information on key age-specific differences in GABAergic neurotransmission that account for the decreased seizure threshold and lack of efficacy of conventional GABA agonists in neonates. Understanding the molecular mechanisms that underlie the differential actions of GABA in immature versus mature neurons has, in turn, helped define targets for novel anticonvulsants in neonates.

In the adult cortex, GABA is the main inhibitory neurotransmitter, in which its activity is essential for maintaining appropriate electrical activity by balancing inputs that are...
triggered by excitatory neurotransmitters such as glutamate. In contrast, in the neonatal cortex, GABA is an excitatory neurotransmitter, where it plays an important role in neuronal development and the activity-dependent wiring of circuits by triggering depolarizing inputs that underlie large-scale spontaneous electrical activity. In immature neurons, GABA-induced membrane depolarization triggers action potentials that directly activate voltage-dependent Ca channels, and indirectly activate N-methyl-D-aspartate receptors. The subsequent GABA-induced elevations of intracellular Ca promote neuronal maturation, and are important for the genesis and maintenance of synaptic connections.

Because the GABA<sub>A</sub> receptor is a ligand-gated Cl<sup>-</sup> channel, the resting level of [Cl<sup>-</sup>]<sub>i</sub>, the E<sub>Cl</sub>, are key determinants of GABA’s effect. Upon binding to GABA<sub>A</sub> receptors, GABA triggers conformational changes in receptor-associated Cl<sup>-</sup> channels that permit either the passive influx or efflux of Cl<sup>-</sup> ions. The directionality of Cl<sup>-</sup> transport depends on the cell’s [Cl<sup>-</sup>]. If the [Cl<sup>-</sup>] is below the E<sub>Cl</sub>, Cl<sup>-</sup> enters the cell and triggers membrane depolarization and inhibition; if the [Cl<sup>-</sup>] is greater than the E<sub>Cl</sub>, Cl<sup>-</sup> enters the cell and triggers membrane depolarization and excitation.

Recent work has shown that the differential action (excitatory vs inhibitory) of GABA in the neonate and adult CNS is due to a difference in the [Cl<sup>-</sup>] of immature versus mature neurons. Compared to mature adult neurons, [Cl<sup>-</sup>] is ~20–40 mM higher in immature neonatal neurons, a difference that is sufficient to shift the action of GABA from inhibition to excitation because the electrochemical driving force for Cl<sup>-</sup> drives the flux of this anion out of the cell when GABA channels are opened. The [Cl<sup>-</sup>] is determined by the Na-K-Cl cotransporter NKCC1, which mediates Cl<sup>-</sup> transport into the cell, and the K-Cl cotransporter KCC2, which mediates Cl<sup>-</sup> transport out of the cell. The NKCC1 and KCC2 cotransporters are related members of the SLC12A electroneutral cation-chloride cotransporter gene family. The cation-chloride cotransporters are intrinsic membrane proteins that transport Cl<sup>-</sup> ions, together with Na and/or K ions, across the plasma membranes of cells throughout the body. They are targets of some of the most commonly used drugs in medicine (the “loop” and thiazide diuretics), and are mutated in several inherited human diseases, including the autosomal-recessive, renal, salt-wasting Gitelman and Bartter syndromes. The stoichiometric coupling and directionality of the ions translocated by the cation-chloride cotransporters results in a secondarily active, nonelectrogenic (electroneutral) transport process that is energetically driven by transmembrane Na and K gradients established by Na<sup>-</sup>-K<sup>+</sup>-adenosine triphosphatase. The cation-chloride cotransporters are divided into 2 main branches, defined by the identity and stoichiometry of
their transported ions, their sensitivity to pharmacological inhibitors, and genetic phylogeny.

Given the large electrochemically favorable inward gradient for Na, the Na-coupled branch comprises the Na-(K)-Cl cotransporters, NKCC (the target of thiazide diuretics), NKCC1, and NKCC2 (the target of Lasix, a furosemide diuretic), which load Cl- ions into the cell to raise [Cl-]i above ECl. Conversely, by coupling the transport of Cl- to an outwardly-directed gradient for K, the 4 different K-Cl cotransporters (KCC1, KCC2, KCC3, and KCC4) primarily transport Cl- ions out of the cell, lowering [Cl-]i below ECl. The relative activities of the Na-(K)-Cl and K-Cl cotransporters determine [Cl-]i in numerous cell types, and their activities are reciprocally regulated by the chloride-sensitive serine-threonine WNK and SPAK/OSR1 kinases.

In neurons, NKCC1 and KCC2 are the cation-chloride cotransporters responsible for determining [Cl-]i. Both bumetanide and furosemide, well-known loop diuretics, are capable of inhibiting the cotrans-chloride transporters in vitro and in vivo. Bumetanide has an approximately 500-fold greater affinity for NKCC1 (K_i of ~ 0.1 µM) than for KCC2 (K_i of ~ 25–50 µM). Furosemide inhibits NKCC1 and KCC2 with equal potency (K_i of ~ 25–50 µM). Therefore, at low doses (2–10 µM) bumetanide is a relatively specific inhibitor of NKCC1. The accumulation of bumetanide in the CNS after systemic administration has not been directly measured, but the drug’s high lipid:water partition coefficient and its documented anticonvulsant effects in both animals and humans in vivo suggest that the drug is able to cross the blood–brain barrier.

A Switch in the Neuronal Expression of NKCC1 and KCC2 Underlies the Excitatory-to-Inhibitory Developmental Shift in GABAergic Signaling

In embryonic and early postnatal life, there is robust neuronal expression of NKCC1, with minimal expression of KCC2. This predominance of inwardly directed Cl- transport via NKCC1 increases [Cl-]i above ECl, such that GABAA receptor activation triggers a passive outflow of Cl- ions that results in membrane depolarization and excitation. Consistent with the important role of NKCC1 for excitatory GABAergic neurotransmission, GABAA receptor-mediated depolarization is not present in NKCC1−/− mice. NKCC1 knockdown with RNA interference promotes precocious inhibitory GABAergic signaling in immature neurons, and excitatory GABAergic signaling can be switched to inhibitory signaling in immature neurons by transgenic overexpression of KCC2.

At birth, KCC2 is expressed at very low levels, and the developmental negative shift in the GABA reversal potential is paralleled by a robust increase in KCC2 expression near the end of the second postnatal week in rats. In humans, KCC2 expression begins at the 40th week after conception. This increase in KCC2 expression, accompanied by the concurrent downregulation in NKCC1 expression, results in a dominance of Cl- export over Cl- import, decreasing [Cl-]i below ECl. Consistent with the important role of KCC2 for establishing inhibitory GABAergic neurotransmission, knockdown of KCC2 in mature hippocampal slices using RNA interference produces a positive shift in E_GABA.

Effect of Bumetanide, an Inhibitor of NKCC1, in Animal Models of Neonatal Seizures

Because the elevated [Cl-]i of immature neurons is due to robust activity of NKCC1, this cotransporter is currently being explored as a target for novel anticonvulsant strategies for neonatal seizures. In theory, inhibition of NKCC1, by reducing [Cl-]i, could reduce the GABA-mediated excitation of immature neonatal neurons, or even possibly convert the GABA response to an inhibitory one. Dzhala et al. were the first to test this hypothesis in a recent seminal paper. Previous groups had established that NKCC1 expression in rats is highest in cortical neurons during the first postnatal week, begins to decrease at post-
Bumetanide and treatment strategies for neonatal seizures

Bumetanide and treatment strategies for neonatal seizures in adults. Conversely, the expression of KCC2 is minimal at birth in rat cortical neurons, is low during the first postnatal week, and then attains an expression level at postnatal Day 14 that is comparable with adults. In rats, the higher expression levels of NKCC1 earlier in development correlate with an increased [Cl\(^-\)]. and with the presence of excitatory GABAergic signaling. Dzhala and colleagues demonstrated that a similar expression pattern is present in the human cortex, with high NKCC1 and low KCC2 expression during the neonatal time period and before the end of the 1st year of life, a time scale that is not significantly different from the scale in the rat when adjusted for the greater length of time required for the development of the human cortex relative to the rodent brain. These data supported the hypothesis that, similar to the rat, GABA is excitatory in immature human cortical neurons, and neonates may be susceptible to seizures due to the excitatory effects of GABA during development. Because NKCC1 is known to establish the elevated levels of [Cl\(^-\)], that underlie excitatory GABAergic signaling in immature neurons, these data predicted that bumetanide, an inhibitor of NKCC1, might be an effective treatment for neonatal seizures.

This hypothesis was tested by Staley in a series of elegant in vitro and in vivo physiological studies. These investigators found that NKCC1 blockade by bumetanide inhibited cortical seizure activity in neonatal rats both in vitro and in vivo, and this inhibition was observed at doses that have already been extensively tested in human neonates in diuresis studies. Specifically, they demonstrated that pharmacological inhibition of NKCC1 by bumetanide: 1) produced a negative shift in E\(_{\text{syn}}\); 2) inhibited GABA-dependent synchronous excitatory activity in the immature hippocampus; 3) suppressed interictal and ictal-like activity in immature hippocampal slices in vitro; and 4) attenuated kainate-induced seizure activity in vivo in neonatal rats. These anticonvulsant effects of bumetanide were shown to be specific, as they were: 1) achieved at low doses that selectively block NKCC1; 2) blocked by antagonists of the GABA\(_{\alpha}\) receptor, indicating that bumetanide is acting through a GABA\(_{\alpha}\) receptor-associated signaling pathway; 3) did not affect epileptiform activity in brain slices from NKCC1\(^-/-\) mice, indicating that inhibition of NKCC1 is the mechanism by which bumetanide exerts its GABA-dependent anticonvulsant effects; and 4) did not depress epileptiform activity in mature neurons, in which the expression of NKCC1 is < 10% of that in neonatal tissue.

Given these promising findings, it seemed reasonable to combine bumetanide, which blocks the excitatory effect of GABA in immature neurons by decreasing [Cl\(^-\)], with phenobarbital, a GABA agonist that opens GABA\(_{\alpha}\) receptor-associated Cl\(^-\) channels. Theoretically, such an increase in GABA-mediated conductance in neurons already targeted by bumetanide would serve to increase shunting inhibition and maximize the anticonvulsant power of the GABA system.

The efficacy of bumetanide, in combination with the GABA-enhancing anticonvulsant phenobarbital, was recently tested for the treatment of recurrent tonic-clonic epileptiform activity in the intact immature hippocampus in vitro. In this study, a low-magnesium model of neonatal seizures in the intact immature hippocampal formation in vitro was employed. Such a model has the benefits of not altering the energy gradient for NKCC1 and preserving longitudinal intrahippocampal connections. Recurrent seizures were induced in the intact hippocampal preparation by a continuous 5-hour exposure to low-magnesium solution, and the anticonvulsant efficacy of phenobarbital, bumetanide, and the combination of these drugs was then studied. Whereas phenobarbital failed to abolish or depress recurrent seizures in 70% of immature hippocampi, phenobarbital in combination with bumetanide abolished seizures in 70% of immature hippocampi, and significantly reduced the frequency, duration, and power of seizures in the remaining 30% of immature hippocampi.

Taken together, these in vitro and in vivo studies suggest that bumetanide, alone or in combination with other drugs such as phenobarbital, might be useful in the treatment of neonatal seizures in humans.

### Bridging the Gap From Animal Models of Neonatal Seizures to Human Subjects

At low concentrations (2–10 μM), bumetanide is a specific inhibitor of NKCC1 and has well-established pharmacokinetic and pharmacodynamic properties in adult humans with few side effects. Because the expression patterns of NKCC1 during development are similar in the human and rat cortex, bumetanide might be useful for the treatment of seizures in human neonates. Bumetanide has been extensively used in both healthy and critically ill human full-term and preterm infants to treat fluid volume overload due to cardiac and/or pulmonary disease, so extrapolation from these studies might help guide the design of any potential pilot studies or clinical trials. However, it will be important to investigate whether the pharmacokinetics of bumetanide are altered by any of the underlying diseases that are responsible for triggering seizures in neonates, because many full-term newborns with refractory seizures have hypoxic-ischemic encephalopathy from perinatal asphyxia, which is often accompanied by multi-organ dysfunction (including hepatic and renal failure); such organ dysfunction can dramatically affect drug metabolism.

Perhaps the best new treatment strategy for neonatal seizures and one that could be easily tested, is a combination regimen that would include bumetanide with a barbiturate like phenobarbital. In the neonatal rat brain, phenobarbital has been rendered a more effective anticonvulsant by coadministering it with bumetanide, which reverses the Cl\(^-\) gradient in immature neurons to a level such that GABA\(_{\alpha}\) potentiation by phenobarbital results in synaptic inhibition. If such a trial were to take place, neonates with persistent seizures despite an initial loading dose of phenobarbital (the current standard of care) could be offered bumetanide along with the second dose of phenobarbital. Continuous electroencephalography monitoring of patients could then be used to determine whether bumetanide reduces seizures compared with controls (those neonates treated with phenobarbital alone). Pilot studies now underway are examining the efficacy of bumetanide, administered with phenobarbital, for the treatment of neonatal seizures (FDA IND No. 101690; see http://www.cure epilepsy.org/research/current.asp).

*Neurosurg. Focus / Volume 25 / September 2008*
The combination of bumetanide and a barbiturate should obviate the need to use the high doses of barbiturate or benzodiazepine that have been associated with significant side effects, such as apoptotic neurodegeneration in the developing brain and late cognitive/behavioral impairment.\textsuperscript{4,5,23,56} Moreover, because of its longstanding safe use in newborns as a diuretic, the low doses of bumetanide that are required to inhibit NKCC1 are not anticipated to produce short- or long-term side effects. However, caution must be exercised as work proceeds, and studies should be performed to determine any potential side effects of inhibiting NKCC1 in the neonatal nervous system, because GABA-mediated excitation is important for neuronal development.\textsuperscript{9} To date, bumetanide-mediated inhibition of NKCC1 in the brain, for periods of time that would far exceed the duration that would be used for the treatment of neonatal seizures, has been shown to have few developmental side effects.\textsuperscript{9,27} These modest but measurable side effects on cortical development must be weighed against the well-known detrimental effects of persistent seizures in the immature brain and the absence of knowledge regarding the developmental effects of NKCC1 inhibition in the setting of such seizures.\textsuperscript{31}

**Excitatory GABAergic Signaling in Adult Epilepsy Syndromes**

Recent studies have also implicated excitatory GABAergic signaling in the genesis of TLE and seizures that occur after ischemic-hypoxic insult in adults.\textsuperscript{37,68} A feature common to these seizure syndromes is that adult neurons adopt transmembrane Cl\textsuperscript{–} gradients that phenotypically resemble those of neonatal neurons, rendering GABA activity excitatory instead of inhibitory.\textsuperscript{32} An increase in the relative abundance and/or activity of NKCC1 in relation to KCC2, which elevates [Cl\textsuperscript{–}], accounts for this pathological shift. For example, neurons from individuals with adult TLE accumulate Cl\textsuperscript{–} in hippocampal neurons to an extent that renders GABA\textsubscript{A} receptor activation excitatory,\textsuperscript{35} apparently because of increased expression of NKCC1 and decreased expression of KCC2.\textsuperscript{51} A human trial of bumetanide is currently underway in adults with medically intractable and surgically unresectable TLE associated with mesial temporal sclerosis or different cortical malformations.\textsuperscript{45}

Bumetanide might also be useful for adult seizures that occur secondary to hypoxic-ischemic encephalopathy or traumatic brain injury (due to brain edema), as NKCC1 is upregulated after both of these insults, and bumetanide has been shown to decrease the excitability that accompanies the increases in neuronal [Cl\textsuperscript{–}], that follow these injuries.\textsuperscript{7,22} For example, in rat models of cerebral hypoxic-ischemic injury, a prolonged rise in [Cl\textsuperscript{–}] in hippocampal neurons renders GABA\textsubscript{ergic} neurons hyperexcitable.\textsuperscript{24,35} This rise in [Cl\textsuperscript{–}], which is completely prevented by bumetanide, is associated with an increase in the expression of the phosphorylated active form of NKCC1.\textsuperscript{55} Bumetanide has also been shown to prevent ischemia-induced hyperexcitability and cell swelling.\textsuperscript{1,70} The activation of glutamate receptors and high extracellular K\textsuperscript{+}, factors that contribute to the pathogenesis of neuronal excitotoxicity, also have been shown to induce the phosphorylation and activation of NKCC1.\textsuperscript{5,71} These data suggest that a pathological increase in NKCC1 activity due to an increase in cotransporter phosphorylation contributes to the ischemia-induced accumulation of intracellular Cl\textsuperscript{–} that triggers the neuronal hyperexcitability, excitotoxicity, and injury to neurons after hypoxic-ischemic insults.\textsuperscript{11,54,78} and suggests that bumetanide might be useful for the treatment of seizures in this clinical context.

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