

● PERSPECTIVE

Pharmacological inhibition of cation-chloride cotransporters for neurological diseases

γ -Aminobutyric acid (GABA) is a major neurotransmitter and plays important roles in both the developing and mature central nervous system (CNS). One way that GABA can act is by binding to fast, ionotropic GABA_A receptors in neurons. The binding of GABA to GABA_A receptors causes a conformational change that opens ion channels, allowing the passage of Cl⁻ ions. In mature adults, intracellular Cl⁻ concentration ([Cl⁻]_i) is low, and the opening of these ion channels triggers influx of Cl⁻ ions, causing hyperpolarization and neuronal inhibition (Blaesse, 2009). However, in the developing nervous system, [Cl⁻]_i is high, and the binding of GABA to GABA_A receptors induces a depolarizing excitatory response (Figure 1). This leads to the stimulation of voltage-dependent Ca²⁺ channels, which is important for proper neuronal proliferation and differentiation in their circuitry development. Gradual changes in [Cl⁻]_i during development, known as the “GABA shift”, determine the strength and polarity of GABA-mediated activity. The precise regulation of [Cl⁻]_i is determined by two major cation-chloride cotransporters (CCCs), the inwardly directed Na-K-2Cl cotransporter (NKCC1) and the Cl⁻ extruding K-Cl cotransporter (KCC2) (Blaesse, 2009). NKCC1 is expressed in a wide range of neurons and glia, but especially in embryonic ventricular zones, which suggests an important role in neuronal proliferation. KCC2 is located in plasma membrane of both somata and dendrites of neurons in various brain regions, including cortical pyramidal neurons, thalamic relay cells, and auditory brainstem neurons (Blaesse, 2009). In embryonic and early postnatal life, neurons show high expression of NKCC1 and low expression of KCC2 (Figure 1). NKCC1 functions in Cl⁻ influx and raises the [Cl⁻]_i during the early development of the CNS, which enables GABA_A receptor-mediated neuronal depolarization and excitation. NKCC1 expression rapidly decreases during the first year of life to levels common in an adult, while KCC2 expression rises (Dzhala et al., 2005). KCC2 functions in Cl⁻ efflux and lowering [Cl⁻]_i, which leads to the dominance of GABA_A receptor-mediated hyperpolarization and inhibition response in the maturing brain (Figure 1).

Dysfunction of the precise balance between NKCC1 and KCC2 activity may be involved in the pathogenesis of several neurological diseases characterized by synaptic inhibition disturbances, such as epilepsy and autism. Because CCCs also function in cell volume regulation, they may contribute to swelling-related neurodegeneration in conditions such as hypoxia and ischemic stroke. Previous attempts to restore proper GABAergic function and neuronal excitability in neurological disease include modifying neurotransmitter levels and release or receptor properties (Kahle et al., 2008), which may have detrimental side effects. New studies illustrate that drugs aimed at restoring normal Cl⁻ homeostasis present advantages by restoring the efficacy of endogenous inhibition and excitation in the neurons rather than actively depressing excitability or imposing inhibition (Kahle et al., 2008). Researchers are actively investigating the efficacy of the NKCC1 inhibitor bumetanide on treating neurological disorders related to GABAergic dysfunction. This review will summarize the usage of bumetanide in clinical trials for brain disorders. Additional discussion focuses on the perspectives of bumetanide in reducing brain damage and accelerating neurological recovery after ischemic stroke.

Blocking NKCC1 activity in epilepsy: Neonatal seizures are epileptic episodes experienced by newborn infants in the first 28 days of life and have devastating effects on brain development (Kahle et al., 2008). In neonates, the activation of Cl⁻ permeable GABA_A excites many neurons, leading to a higher propensity for seizures (Kahle et al., 2008). Anti-epileptic drugs that target GABA_A receptors (such as GABA_A receptor blockers phenobarbital and benzodiazepines) are effective in adults in whom [Cl⁻]_i is low and there is a passive Cl⁻ influx. However, these drugs are ineffective and perhaps even detrimental to neonates who have a high intracellular Cl⁻ accumulation mediated through the action of NKCC1. A preclinical study from the Staley lab (Dzhala et al., 2005)

hypothesized that alteration of Cl⁻ ionic homeostasis, a basis for GABA-mediated excitation in the immature brain, by NKCC1 blocker bumetanide may inhibit seizure activity. Seizure was induced in postnatal days 9–10 (P9–10) rat pups by subcutaneous injection of kainic acid (2 mg/kg) (Dzhala et al., 2005). The rat pups experienced behavioral abnormalities 10–20 minutes later and ictal EEG patterns 20–80 minutes later. Subsequently, the rats received 0.1–0.2 mg/kg bumetanide 10 minutes after kainic acid injection. They showed a decrease in the mean duration of a single ictal episode from 96.8 ± 8.6 seconds to 32 ± 3.9 seconds. Also, the mean interval between recurrent seizures increased from 323 ± 23 seconds to 360.1 ± 68.6 seconds (Dzhala et al., 2005). These results showed that the application of bumetanide attenuated kainite-induced seizure activity in rat pups.

In 2013, the first pilot clinical study looking at the efficacy of bumetanide on the reduction of seizure frequency in human patients was performed (Eftekhari et al., 2013). The study evaluated 3 patients (31–37 years old) with drug-resistant temporal lobe epilepsy (TLE) (Eftekhari et al., 2013). Bumetanide was administered 2 mg daily, and patients were visited weekly to monitor their symptoms and quality of life. Over a period of 4 months, the first patient showed a reduction in the number of days of which the patient experienced a seizure (seizure-day frequency) by 68%. The second patient had seizure-day frequency reduction by 84%. The third patient exhibited seizure-day frequency reduction by 75%. The results in this study revealed that bumetanide can be a suitable substitute for surgical treatment of patients with nonresponsive TLE without major side effects. However, more extensive studies are needed to elucidate the exact role of bumetanide in seizure control.

Another clinical study named NEMO assessed dose and feasibility of intravenous bumetanide as an add-on to phenobarbital for treatment of neonatal seizures (Pressler et al., 2015). Fourteen newborn infants with electrographic seizures due to hypoxic ischemic encephalopathy and who were unresponsive to a loading-dose of phenobarbital, received an additional dose of phenobarbital and one of four (0.05, 0.1, 0.2, or 0.3 mg/kg) bumetanide doses (Pressler et al., 2015). Five infants met EEG criteria for seizure reduction after a first dose of bumetanide. Two of the five continued to experience seizure reduction 3–4 hours later. The remaining infants required the administration of additional antiepileptic drugs due to clinically unacceptable seizure frequency. In addition, three of the eleven surviving infants had hearing impairments within the first month of life (Pressler et al., 2015). This is likely due to disruption of NKCC1-dependent electrolyte homeostasis in the endolymph of the inner ear, resulting in hair cell degeneration (Wright, 2009). These data suggest that the treatment might increase the risk of hearing loss without evidence for improving seizure control. The trial highlights the challenges posed for the trials of antiepileptic drugs in newborn infants, and the necessity for innovative pharmacokinetics of new NKCC1 blockers.

Blocking NKCC1 activity in autism: It is currently believed that GABAergic signals are altered in autism and the underlying mechanisms may involve the polarity shift of GABAergic actions due to altered [Cl⁻]_i. An encouraging pilot open trial was conducted in which bumetanide (1 mg daily) was administered over a 3 month period to 5 children with autistic disorder. The results showed significant improvements in behavior and no side effects (Lemonnier and Ben-Ari, 2010). Following this, a study was conducted to test chronic administration of bumetanide (3 months, 1 mg daily) in 60 children (3–11 years old) with autism/Asperger (A/A) syndrome. Bumetanide reduced the severity of A/A with few side effects (Lemonnier et al., 2012). Although the direct evidence that bumetanide exerts its actions by reducing [Cl⁻]_i in neurons in humans is lacking, its amelioration of symptoms invites more interest.

Perspective of NKCC1 inhibition in acute brain injury: The use of bumetanide may prove useful not only in neurological disorders directly linked to GABAergic dysfunction, but also in disorders that cause secondary disturbances in neuronal ionic homeostasis, such as ischemia. A recent study demonstrated that [Cl⁻]_i of hippocampal neurons increased in response to oxygen-glucose deprivation (OGD) in two distinct phases: briefly during OGD and persistently ~1 hour after reoxygenation (Pond et al., 2006). The administration of bumetanide was incapable of reducing the initial rise in [Cl⁻]_i during OGD (Pond et al., 2006). However, bumetanide (10 μM) was effective in blocking the secondary rise of [Cl⁻]_i at a concentration that selectively blocks NKCC1.

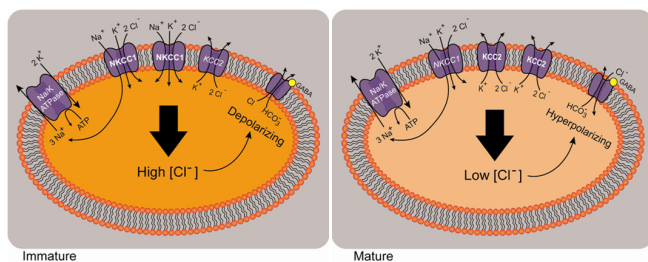


Figure 1 NKCC1 and KCC2 regulate intracellular chloride concentrations in immature and mature neurons.

Left panel: The high expression of NKCC1 protein in the immature, developing brain leads to high $[Cl^-]_i$ and GABA-mediated depolarization. Right panel: As the brain matures, NKCC1 expression is downregulated and KCC2 expression is upregulated. The higher expression of KCC2 protein reduces $[Cl^-]_i$ and hyperpolarizes mature neurons upon GABA_A activation. Balanced function of NKCC1 and KCC2 in specific neurons dictates tight control of $[Cl^-]_i$ (adapted from Blaesse et al., 2009).

This study suggests that OGD induces a $[Cl^-]_i$ increase during reoxygenation as a result of the enhanced activity of NKCC1. Jantzie et al. (2015) demonstrated that NKCC1 is highly expressed in oligodendrocytes and increases vulnerability to hypoxia-ischemia mediated white matter injury. Bumetanide treatment showed attenuation of white matter loss and neuronal damage. Therefore, NKCC1-mediated Cl^- influx may contribute to both neuronal and non-neuronal brain cell damage. It has been well documented in animal studies that ischemia-induced stimulation of NKCC1 plays an important role in cell death after ischemic stroke.

A recent report by Begum et al. (2015) investigated the signaling pathway underlying NKCC1 activation in ischemic stroke. The Cl^- -sensing WNK (with no lysine) and the Ste20/SPS1-related proline-alanine-rich protein kinase/oxidative stress responsive 1 (SPAK/OSR1) serine-threonine kinases comprise an evolutionarily conserved signaling pathway that regulates the activities of NKCC1 to control cell volume and epithelial ion transport. Transgenic knockout of WNK3 showed less NKCC1 stimulation, reduced brain damage, and accelerated recovery of neurological function in ischemic mice (Begum et al., 2015). Changes of CCCs in traumatic brain injury (TBI) should be explored. It is becoming clear that TBI leads to several short- and long-term changes in neuronal circuits which ultimately lead to an imbalance of cortical excitation and inhibition. Therefore, maintaining the delicate homeostasis between GABAergic and glutamatergic firing is crucial to normal neurological function and recovery after TBI. CCCs may contribute to alteration of Cl^- homeostasis in traumatic brain injury.

Effects of long-term NKCC1 blockage: The long-term effects of pharmacological inhibition of NKCC1 on cortical synapse formation remain unclear. Studies have shown that GABA-mediated depolarization can regulate the development of excitatory synapses in the developing cortex *in vivo*. A recent study reported that disruption of GABA signaling through bumetanide in the developing brain resulted in permanent decreases in excitatory synaptic transmission and sensorimotor gating deficits (Wang and Kriegstein, 2011). Intraperitoneal injections of bumetanide (0.2 mg/kg) was given to pregnant mice prenatally and their pups postnatally. Electrophysiological experiments showed that blocking NKCC1 with bumetanide during a critical period leads to lasting changes in cortical excitatory transmission (Wang and Kriegstein, 2011). Bumetanide was injected from E15 to P7, and subsequent imaging at postnatal 4 weeks demonstrated fewer primary and secondary dendrites, as well as decreased dendrite length, dendrite volume, branch levels, branch points, and number of dendrite segments and terminal points (Wang and Kriegstein, 2011). In addition, a broad screen for differences in various fundamental behavioral domains showed decreased strength and motor coordination. Later at P7–P14, these mice showed functional impairments in sensorimotor gating functions (Wang and Kriegstein, 2011). The results demonstrate that NKCC1 plays an important role in neuron differentiation and brain development. Treatment with bumetanide during a critical early period results in developmental delay and long-term impairment in sensorimotor gating. These studies raise the concerns for long-term usage of bumetanide to treat the disorders in immature brains in light of significant role of NKCC1 in brain development.

In summary, new clinical evidence suggests that pharmacological blockade of CCCs may improve $[Cl^-]_i$ homeostasis dysregulation and restore endogenous GABAergic inhibition and glutamatergic excitability in neurological diseases with disturbed neuronal circuits, such as epilepsy and autism. The usage of the current CCC-targeting drugs in the preclinical studies, such as bumetanide, shows their potentials to reduce brain damage and accelerate neurological recovery in acute brain disorders that have secondary ionic disturbances including ischemic

stroke. However, recent investigation of bumetanide pharmacokinetics shows rapid elimination and poor brain penetration (Brandt, 2010), which may lead to negative side effects, such as clinical deficits in hearing and developmental delay in mice. Additional investigation of the long-term effects of the current CCC targeting drugs in the immature brain is warranted. Developing more selective and potent inhibitors of the CCCs with less adverse side effects is needed. Better understanding of roles of NKCC1 in the neurological diseases may lead to new alternative and more effective therapies.

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Accepted: 2015-11-10

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doi: 10.4103/1673-5374.172313 <http://www.nrronline.org/>

Nepomuceno R, Sun D (2015) Pharmacological inhibition of cation-chloride cotransporters for neurological diseases. *Neural Regen Res* 10(12):1924-1925.

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