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The role of KCC2 in hyperexcitability of the neonatal brain

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Abstract

Background: The hyperpolarizing activity of γ -aminobutyric acid A (GABA_A) receptors depends on the intracellular chloride gradient that is developmentally regulated by the activity of the chloride extruder potassium (K) chloride (Cl) cotransporter 2 (KCC2). In humans and rodents, KCC2 expression can be detected at birth. In rodents, KCC2 expression progressively increases and reaches adult-like levels by the second postnatal week of life. Several studies report changes in KCC2 expression levels in response to early-life injuries. However, the functional contribution of KCC2 in maintaining the excitation-inhibition balance in the neonatal brain is not clear. In the current study, we examined the effect of KCC2 antagonism on the neonatal brain activity under hyperexcitable conditions *ex vivo* and *in vivo*.

Methods: *Ex vivo* electrophysiology experiments were performed on hippocampal slices prepared from 7–9 days-old (P7-P9) male rats. Excitability of CA1 pyramidal neurons bathed in zero-Mg²⁺ buffer was measured using single-unit extracellular (loose) or cell-attach protocol before and after application of VU0463271, a specific antagonist of KCC2. To examine the

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functional role of KCC2 *in vivo*, the effect of VU0463271 on hypoxia-ischemia (HI)-induced ictal (seizures and brief runs of epileptiform discharges - BREDs), and inter-ictal spike and sharp-wave activity was measured in P7 male rats. A highly sensitive LC-MS/MS method was used to determine the distribution and the concentration of VU0463271 in the brain.

Results: *Ex vivo* blockade of KCC2 by VU0463271 significantly increased the frequency of zero-Mg²⁺-triggered spiking in CA1 pyramidal neurons. Similarly, *in vivo* administration of VU0463271 significantly increased the number of ictal events, BREDs duration, and spike and sharp-wave activity in HI rats. LC-MS/MS data revealed that following systemic administration, VU0463271 rapidly reached brain tissues and distributed well among different brain regions.

Conclusion: The results suggest that KCC2 plays a critical functional role in maintaining the balance of excitation-inhibition in the neonatal brain, and thus it can be used as a therapeutic target to ameliorate injury associated with hyperexcitability in newborns.

Keywords

KCC2; chloride transporter; newborn; seizures; hypoxia-ischemia; hyperexcitability

1. Introduction

The neonatal age is the period with the highest probability of seizure occurrence [1]. One of the factors that contribute to the increased susceptibility of neonatal brain to seizures is the excitatory activity of γ -aminobutyric acid (GABA), a major inhibitory neurotransmitter in the mature brain. The excitatory action of GABA during the early developmental period is due to higher intracellular chloride levels, which is primarily regulated by two cation-chloride cotransporters: Na-K-Cl Co-transporter 1 (NKCC1) and K-Cl cotransporter 2 (KCC2) [2]. The levels of both, the chloride importer NKCC1 and the chloride extruder KCC2, are developmentally regulated. Immature neurons express high levels of NKCC1 and low levels of KCC2. Progressively, as neurons mature, the levels of KCC2 increases switching the action of GABA from excitatory to inhibitory.

The contribution of cation-chloride cotransporters to hyperexcitability in the neonatal brain is not fully understood. The prevailing hypothesis is that chloride uptake via NKCC1 is the major mechanism promoting hyperexcitability [3–5]. Support for this hypothesis comes from studies demonstrating that neonatal seizures enhance NKCC1-mediated chloride uptake, which in turn results in depolarizing responses to GABA and hyperexcitability [6–8]. The diuretic drug bumetanide, an inhibitor of NKCC1, has been widely used to suppress neonatal epileptiform activity [3]. Although dampening NKCC1 activity with bumetanide attenuated electrographic seizures, it did not provide complete protection [3, 4]. In the neonatal brain, chloride extrusion is enhanced in response to “mild” seizures by a rapid redistribution of KCC2 transporters from an intracellular compartment to the plasma membrane [9]. However, severe or prolonged seizures, similar to what have been observed following neonatal hypoxia-ischemia (HI), result in KCC2 cleavage and inactivation concomitantly with a rise in intracellular chloride [5–7, 10, 11]. These findings point to the existence of an endogenous homeostatic mechanism to counteract seizure generation and

also suggests that maintaining/enhancing KCC2 activity might be a powerful strategy to suppress seizures and brain damage [12, 13].

In adult animal models, inhibition of KCC2 activity by a selective pharmacological inhibitor, VU0463271, increased brain hyperexcitability of the brain resulting in increased spiking activity and seizures [14, 15]. These observations suggest that KCC2 function is critical to maintain normal inhibitory function in the adult brain. However, despite increasing evidence, the contribution of KCC2 to the balance of excitation-inhibition in the neonatal brain is not fully understood. Therefore, in the current study we examined the effects of the KCC2 inhibitor VU0463271 on neonatal brain excitability using *ex vivo* and *in vivo* methods. Since 60% of neonatal seizures are associated with hypoxic-ischemic injury [16], the *in vivo* studies were performed in an animal model of neonatal hypoxic-ischemic encephalopathy.

2. Materials and methods

2.1. Animals

Animal experiments were performed in accordance with the NIH guidelines for the care and use of laboratory animals, and according to the protocol approved by the Institutional Animal Care and use Committee (IACUC) of the University of Colorado Anschutz Medical Campus (UCAMC). All efforts were made to reduce the number of animals used and animal suffering. Timed pregnant Sprague-Dawley rats were received at gestational day 14 from Charles River Laboratories. Since expression of KCC2 is sexually dimorphic [2], and because males are more severely affected by HI and have worse neurologic outcomes than females with hypoxic-ischemic injury [17], all the experiments in the current study were performed on male pups only. The pups were selected randomly for a specific drug treatment on the day of the experiment, and whenever possible, pups from each litter were divided equally among the treatment groups.

2.2. Brain slice preparation for patch-clamp experiments

P7-P9 rat pups were briefly anesthetized with isoflurane and quickly decapitated, and their brains rapidly removed. Horizontal brain slices (300 μm) were sectioned at 4°C in a prechilled solution containing (in mM): sucrose 260, D-glucose 10, NaHCO_3 26, NaH_2PO_4 1.25, KCl 3, CaCl_2 2, MgCl_2 2, using a tissue slicer (Leica VT 1200S, Buffalo Grove, IL). Brain slices were immediately incubated for 45 minutes at 37°C in a solution containing (in mM): NaCl 124, D-glucose 10, NaHCO_3 26, NaH_2PO_4 1.25, KCl 4, CaCl_2 2, MgCl_2 2 prior to use in electrophysiology experiments, which were done at room temperature. During incubation, slices were constantly perfused with a gas mixture of 95 vol% O_2 and 5 vol% CO_2 .

2.3. Slice electrophysiology *ex vivo* experiments

The external solution for patch-clamp electrophysiology experiments consisted of (in mM): NaCl 125, D-glucose 10, NaHCO_3 26, NaH_2PO_4 1.25, KCl 4, CaCl_2 0.25. This solution was equilibrated with a mixture of 95 vol% O_2 and 5 vol% CO_2 for at least 30 minutes with a resulting pH of about 7.4. This modified aCSF solution was chosen to promote neuronal excitability [14]. The internal solution consisted of (in mM): potassium-D-gluconate 130,

EGTA 5, NaCl 4, CaCl₂ 0.5, HEPES 10, pH 7.3. Glass micropipettes (Sutter Instruments O.D. 1.5 mm) were pulled using a Sutter Instruments model P-1000 and fabricated to maintain an initial resistance of 3–4 MΩ. Neuronal membrane responses were recorded using a Multiclamp 700B amplifier (Molecular Devices, Foster City, CA). To assess excitability of CA1 hippocampal pyramidal neurons, we used a single-unit extracellular (loose) or cell-attach protocol with a relatively low membrane resistance (<100 MΩ) at the holding membrane potential of 0 mV [18]. Voltage current commands and digitization of the resulting voltages and currents were performed with Clampex 8.3 software (Molecular Devices, Foster City, CA) running on a compatible computer. Resulting current traces were analyzed offline using MiniAnalysis software (Synaptosoft, Inc., Decatur, GA).

2.4. Electrode implantation

Electrodes to record EEG were implanted on P6 rat pups according to our previously published protocol [19]. Briefly, the pups were anesthetized with isoflurane (2–4% for induction and 1–1.5% for maintenance) and a silver wire was implanted in the parietal cortex of each hemisphere (A-M Systems, Carlsborg, WA). The active electrode in each hemisphere was referenced to a separate silver electrode positioned near lambda in the same hemisphere. After the surgery, rats were returned to the dam and treated with analgesic (0.1 mg/kg buprenorphine hydrochloride) once every 12 hours for 48 hours.

2.5. Hypoxia-ischemia induction

HI was induced at P7 according to Rice-Vannucci protocol as described in detail in our previous publication [19]. Briefly, the right common carotid artery of the pup was double ligated with 4–0 nylon Dermalon sutures under isoflurane anesthesia (2–4% for induction and 1–1.5% for maintenance). Two hours after ligation, pups were subjected to 2 h hypoxia (8–8.3% oxygen concentration monitored using an oxygen sensor) in a chamber whose temperature and humidity were maintained at 36.5°C and 60–70%, respectively. Following HI-induction, the pups were treated with analgesic (0.1 mg/kg buprenorphine hydrochloride) once every 12 hours for 48 hours.

2.6. Video-EEG monitoring

Synchronized, time-locked video and EEG signals were recorded using the Stellate Harmonie system (Natus Medical, San Carlos, CA). EEG signals were digitized at 1000 Hz and stored on a hard disk for offline analysis. A 30 min baseline recording was acquired at P7 before any procedures to induce HI were performed. The pups were continuously monitored by video and EEG during hypoxia period (2 h).

2.7. Seizure, spikes, and sharp-waves scoring

Video-EEG records were manually analyzed to identify seizures defined by an EEG pattern that differed from background in either amplitude, frequency, or both, evolved over time, and contained spikes or sharp-waves lasting for 10 s or more and were associated with a change in the rat's behavior. An EEG pattern similar to that observed during seizure with duration of >2 but less than 10 s was classified as brief runs of epileptiform discharges (BREDs). Seizures combined with BREDs were classified as ictal events. Spikes and sharp-

waves were defined as brief (20–200 ms), high amplitude, sharply contoured waveforms distinct from background EEG patterns.

2.8. Drug treatment

The KCC2 antagonist, VU0463271 (Tocris, Bio-Techne, Minneapolis, MN, USA), was freshly prepared before use by dissolving it in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). Equivalent volume of experimental drug and vehicle (DMSO) were administered by intraperitoneal (i.p.) route. The first dose (100 µg/kg body weight) was administered 5 minutes before the initiation of hypoxia and three additional doses were administered every 30 minutes (total 4 doses given at 30 minutes of interval). This frequency of administration was chosen due to the rapid metabolism of VU0463271 [20]. All the injections occurred during the 2 h hypoxia period.

2.9. LC-MS/MS analysis

ML-077 (VU0255011; internal standard) was purchased from AOBIOUS, Inc. (Gloucester, MA). Acetonitrile, formic acid, methyl tert-butyl ether (MTBE), nitric acid, and sodium hydroxide were purchased from Thermo Fisher Scientific (Waltham, MA). Four doses of VU0463271 (100 µg/kg, 30 minutes apart, similar to the application scheme for the EEG recording experiments) were administered to P7 rats. Pups were euthanized under isoflurane anesthesia 15 minutes after the last dose. Pups were wiped thoroughly with 70% ethanol to remove any urine, feces, or drug present on the surface of the skin before blood was collected through heart puncture, followed by a transcardial perfusion with cold, sterile phosphate buffered solution (PBS). After perfusion, whole hippocampus, part of cortices, and the remaining brain (containing cerebellum, but minus olfactory bulb, pons, and medulla) were collected, weighed, frozen on dry ice. To obtain serum, the blood was left at room temperature for 20 minutes and spun at 3000 rpm for 10 minutes. All the tools that came in contact with tissues were either replaced or rinsed in 1 N nitric acid, 1 L double distilled water, DMSO, and 1 L double distilled water in between animals. Brain tissues and serum were stored at –80°C until further use. To prepare samples for LC-MS/MS analysis, cortex (sensory and motor cortex), hippocampus, the remaining brain tissue (all about 30 mg), and serum samples (30 µL aliquots) were diluted with water, spiked with internal standard ML-077, and homogenized (serum samples did not require homogenization) by shearing with a pipette followed by sonication for 5 min. Samples were mixed with 60 µL of 0.1N NaOH, vortexed, bath sonicated for 5 min, and mixed with 1 mL of MTBE. Sample solutions were vortexed, bath sonicated for 5 min and then spun down at 10,000 rcf for 5 min. The supernatant was collected into a fresh microcentrifuge tube capped with hole-punched lids and dried in a Savant™ Speedvac™, Model SC100. Dried samples were stored at –20°C until analysis. Immediately prior to LC-MS/MS analysis, dry samples were reconstituted in water and loaded into 96-well microtiter plates. A Prominence HPLC (Shimadzu, Kyoto, Japan) coupled to a QTrap 4500 mass spectrometer (AB Sciex, Framingham, MA) was used for the analysis. The mass spectrometer was operated in the ESI⁺ mode and all settings were optimized by manually tuning to infused standard solutions. The MS/MS transitions selected for analysis were m/z 382.9 → m/z 229.0 for VU0463271 and m/z 357 → m/z 229 for ML-077. Data was collected in the multiple reaction mode (MRM). Analytical separation was achieved with a Phenomenex Kinetex C18 column [2.1 ×

100 mm, 2.6 m] (Phenomenex, Torrance, CA). The column was held at 40°C and eluted at 0.6 mL/min with a gradient of 0.1% formic acid (A) and 0.1% formic acid in 9:1 acetonitrile:water (B). Chromatographic separation was achieved with a linear gradient (time, % of solvent B): 0–3.9 min, 5–33% B; 3.9–4.5 min, 33–66% B; 4.5–6.5 min, 66–94% B; 6.5–7 min, 94–5% B; and then isocratic for 3 minutes at 5% B to re-equilibrate the column. The first three minutes of eluent was diverted to waste to reduce contamination of the front end of the mass spectrometer. Under these conditions, VU0463271 and ML-077 co-elute due to their structural similarity.

2.10. Statistical analysis

GraphPad Prism 5 statistical software (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. A Student's t-test was used to compare two sets of data. Differences were considered significant at $p < 0.05$. The results are presented as the mean $\pm$ standard deviation (SD).

3. Results

3.1. VU0463271 augments spiking frequency of CA1 hippocampal neurons

To investigate the functional impact of KCC2 in controlling hyperexcitability within the neonatal brain, we first used *ex vivo* electrophysiological recordings in hippocampal tissue obtained from P7-P9 rat pups. Slice recordings from pyramidal cells from the CA1 region of hippocampus were obtained using a cell-attach mode in a modified (high K^+ and zero Mg^{2+}) aCSF solution in order to promote cell excitability and to test if application of VU0463271 (10 μM) will further exacerbate epileptiform activity [14]. This experiment showed that incubation of hippocampal slices with zero- Mg^{2+} buffer triggered spontaneous spike firing (Fig. 1A) and that addition of VU0463271 to the bathing media produced an exacerbation of neuronal firing (0.92 ± 0.71 Hz vs. 1.56 ± 1.28 Hz; Fig. 1B), suggesting that blockade of KCC2 with VU0463271 augments neuronal activity (paired t test, $t_7 = 2.20$, $p = 0.032$; $n = 8$ neurons in each group, Fig. 1C). These *ex vivo* observations suggest that pharmacological blockade of KCC2 activity by VU0463271 promotes the augmentation of neuronal activity.

3.2. VU0463271 exacerbates hyperexcitability associated with hypoxic-ischemic injury

To explore the functional role of KCC2 *in vivo*, the effect of VU0463271 on HI-induced hyperexcitability was examined as described previously [19]. The first dose of VU0463271 was administered 5 minutes before the initiation of hypoxia and three additional doses were administered every 30 minutes during hypoxia period. Administration of VU0463271 exacerbated seizure activity associated with hypoxia exposure. The types of ictal events identified in the EEG recordings corresponding to the HI period included both full-blown seizures (EEG seizures with a behavioral correlate lasting 10 seconds or more) and brief runs of epileptiform discharges (BREDs) characterized by EEG patterns that were associated with a behavioral correlate that lasted 3–9 seconds [19]. A quantitative analysis of the results from the inspection of the EEG recordings obtained under HI conditions indicated that pharmacological application of VU0463271 resulted in KCC2 inhibition that in turn resulted in a significant increase in hyperexcitability (Fig. 2). This was indicated by a significant increase in the number of ictal events (ictal activity) (Fig. 3; unpaired t test, $t_8 = 3.08$ and $p =$

0.015; $n = 5$ pups in each group). A separate analysis of seizure and BREDs events showed that the average numbers were higher in VU0463271 injected HI rats when compared to vehicle treated HI rats, but the difference did not reach statistical significance (Fig. 3; unpaired t test, $t_8 = 2.28$ and $p = 0.051$ for seizure numbers, $t_8 = 2.3$ and $p = 0.0502$ for BREDs number, $n = 5$ pups in each group). In terms of duration, BREDs duration showed a significant increase (Fig. 3; unpaired t test, $t_8 = 2.6$ and $p = 0.03$; $n = 5$ pups in each group) but no significant change in ictal and seizure duration was observed between the two treatment groups (Fig. 3; unpaired t test, $t_8 = 1.6$ and $p = 0.14$ for ictal duration and $t_8 = 0.7$ and $p = 0.5$ for seizure duration, $n = 5$ pups in each group).

To quantify spike and sharp-wave activities, EEG epochs of ten minutes selected from baseline recordings and inter-ictal recordings obtained after the 1st and 2nd administration of vehicle or VU0463271 were manually scanned. In order to normalize the counts detected after the administration of vehicle or VU0463271 with the activity detected during baseline conditions, the numbers of spikes and sharp-waves counted under baseline conditions was deducted from the total count obtained after vehicle or drug administration to obtain the final count shown in figure 4. The spike and sharp-wave activity observed under hypoxic conditions was significantly higher in VU0463271 administered rats than in vehicle treated rats (Fig. 4; unpaired t test, $t_8 = 3.41$ and $p = 0.009$; $n = 5$ pups in each group), suggesting that pharmacological blockade of KCC2 also exacerbates inter-ictal hyperexcitability. Taken together, these observations suggest that KCC2 activity is required to maintain normal E/I balance in the neonatal brain.

3.3. Distribution and concentration of VU0463271 in the neonatal brain

To determine the amount of VU0463271 that reached the brain and can be associated to the abovementioned effects, a separate group of P7 rats were dosed with VU0463271 in a similar way as described for the EEG studies (four intraperitoneal doses of 100 $\mu\text{g/kg}$ given at 30 minutes interval). Following VU0463271 administration, samples of cortex, hippocampus and the remaining brain tissue in addition to serum samples were analyzed by LC-MS/MS. With the use of an internal standard, the lower limit of quantitation for VU0463271 on column was calculated to be 100 amol on column (accuracy = 107.4%; %RSD = 11.14%). Under these conditions of detection, VU0463271 concentrations in cortex, hippocampus, the remaining brain and serum were 24.57 ± 11.95 , 15.69 ± 13.09 , 14.56 ± 9.51 , and 18.15 ± 14.47 ng/g tissue, respectively (Fig. 5), suggesting rapid absorption and good brain distribution of the drug relative to serum levels. The LogP for VU0463271 was computed to be 3.32 using ACD/Percepta PhysChem Profiler (Advanced Chemistry Development, Toronto, Ontario, Canada).

4. Discussion

In humans, expression of the chloride transporter, KCC2, begins during the mid-fetal period and reaches adult-like levels by the sixth month of postnatal life [21]. At birth, strong KCC2 immunoreactivity can be detected in neurons of all cortical layers [21]. In mice and rats, KCC2 labeling can be first detected by the end of embryonic development and peaks during the second postnatal week [22, 23]. Although it appears that KCC2 is not fully operational

during embryogenesis, pyramidal neurons from rat hippocampus show KCC2-dependent extrusion of chloride ions that controls the chloride gradient as early as five days post-birth [24] and becomes fully operational by P10-P15 [25]. Besides KCC2 levels of expression, there are a number of other factors such as phosphorylation of various amino acids in the C-terminus, which may limit KCC2 activity during early development (for review see [26]). Despite this, there is no consensus about the contribution of KCC2 to the control of brain excitability during pathological events. In the current study, we show that even though KCC2 expression might be limited in the developing brain, this low level of expression plays a significant functional role in limiting overexcitation under stressed conditions.

Hypoxia-ischemia, the most common cause of seizures in newborns, is associated with down regulation of KCC2 levels. Preventing KCC2 loss improves outcomes, implying its functional significance during early development. Robinson et al. [27] observed reduced levels of KCC2 in postmortem cerebral samples of preterm infants with white matter injury. Similar observations were made by Jantzie et al. [13] in samples from the CA3 region of hippocampus obtained from an animal model of prenatal hypoxic-ischemic injury. In neonatal rats exposed to hypoxia, Cleary et al. [28] observed a transient increase in KCC2 protein levels in hippocampus 1 hour after hypoxia and a decrease in cortex 24 hours post-injury. Ma and colleagues [29] showed that hypoxia-ischemia in neonatal rats was associated with KCC2 loss in hippocampus and cortex, and diazepam treatment prevented this loss, reduced brain injury, and improved long-term behavioral outcomes. Similarly, in neonatal mice, a significant reduction in KCC2 levels was observed in ischemic hemisphere and preventing this loss by a TrkB receptor antagonist was associated with improved efficacy of phenobarbital against ischemia-induced neonatal seizures [30].

The results presented here provide direct evidence to further support a role of KCC2 in the neonatal brain since a selective inhibitor of KCC2, VU0463271, exacerbated hyperexcitability in both *ex vivo* (zero-Mg²⁺ buffer) and *in vivo* (neonatal hypoxia-ischemia) seizure models. Specifically, VU0463271 augmented spiking frequency of CA1 hippocampal neurons in slices bathed in zero-Mg²⁺ buffer and increased the frequency of ictal events and inter-ictal spike and sharp-wave activities associated with hypoxic-ischemic injury. Similar effects of KCC2 antagonism by VU0463271 were also observed in adult animal models. Infusion of VU0463271 directly into the dorsal hippocampus of adult male mice resulted in behavioral arrest that was associated with rhythmic spiking on hippocampal EEG [14]. In the same study, application of VU0463271 converted repetitive seizure-like events induced by zero-Mg²⁺ into persistent, unremitting seizure-like activity in entorhinal cortex slices obtained from 3–5 weeks male mice. In another study, addition of VU0463271 increased frequency of 4AP-induced interictal spiking in entorhinal cortex slices obtained from adult male rats [15].

VU0463271 has 100-fold selectivity for KCC2 over NKCC1 with no activity on a number of G-protein coupled-receptors, ion channels and transporters [20]. We used a highly sensitive LC-MS/MS method to determine brain concentrations of VU0463271 that might have contributed to its brain effects. Following systemic administration, the drug rapidly reached brain tissues and distributed well among different brain regions and reached similar brain:serum concentrations that ranged from 0.8–1.4 ng/g tissue. The high brain distribution

ration of the compound is consistent with its high lipophilicity (Log P of 3.32; Computed by ACD/Percepta PhysChem Profiler).

One of the caveat of the current study is that we examined the role of KCC2 only in neonatal males as male pups are more severely affected by HI and have worse neurologic outcomes than females after a hypoxic-ischemic injury [17]. However, the timeline of KCC2 development is different in male and female rodents. In females, KCC2 level rises earlier in development with a concomitant increase in hyperpolarizing GABA activity [2]. Since the development of KCC2 occurs differently in males and females, it is possible that an even more pronounced effect of VU0463271 could be observed in neonatal female rats, thus similar study would be required to understand functional significance of KCC2 in the neonatal female brain.

5. Conclusions

Studies in humans and animal models suggest that neonatal seizures contribute to brain damage and worsen adverse neurologic outcomes [31, 32]. In the past several years, significant efforts have been devoted to developing new therapies to improve the treatment of neonatal seizures. These efforts have been mostly focused on exploring the role of NKCC1 in hyperexcitability, however the results are not fully satisfactory [3, 4, 33]. Our study suggests that enhancing KCC2 activity could be a powerful strategy to counteract adverse effects of hyperexcitability on neonatal brain.

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References

- [1]. Hauser WA, Annegers JF, Kurland LT, Incidence of epilepsy and unprovoked seizures in Rochester, Minnesota: 1935–1984., *Epilepsia*, 34 (1993) 453–468. [PubMed: 8504780]
- [2]. Galanopoulou AS, Sexually dimorphic expression of KCC2 and GABA function, *Epilepsy Res*, 80 (2008) 99–113. [PubMed: 18524541]
- [3]. Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, Delpire E, Jensen FE, Staley KJ, NKCC1 transporter facilitates seizures in the developing brain, *Nat Med*, 11 (2005) 1205–1213. [PubMed: 16227993]
- [4]. Dzhala VI, Brumback AC, Staley KJ, Bumetanide enhances phenobarbital efficacy in a neonatal seizure model, *Annals of Neurology*, 63 (2008) 222–235. [PubMed: 17918265]
- [5]. Khanna A, Walcott BP, Kahle KT, Limitations of current GABA agonists in neonatal seizures: Toward GABA modulation via the targeting of neuronal Cl(–) transport, *Front Neurol*, 4 (2013) 78–86. [PubMed: 23805124]
- [6]. Jin X, Huguenard JR, Prince DA, Impaired Cl(–) extrusion in layer V pyramidal neurons of chronically injured epileptogenic neocortex, *J Neurophysiol*, 93 (2005) 2117–2126. [PubMed: 15774713]

- [7]. Pathak HR, Weissinger F, Terunuma M, Carlson GC, Hsu FC, Moss SJ, Coulter DA, Disrupted dentate granule cell chloride regulation enhances synaptic excitability during development of temporal lobe epilepsy, *J Neurosci*, 27 (2007) 14012–14022. [PubMed: 18094240]
- [8]. Barmashenko G, Hefft S, Aertsen A, Kirschstein T, Kohling R, Positive shifts of the GABAA receptor reversal potential due to altered chloride homeostasis is widespread after status epilepticus, *Epilepsia*, 52 (2011) 1570–1578.
- [9]. Galanopoulou AS, Dissociated gender-specific effects of recurrent seizures on GABA signaling in CA1 pyramidal neurons: role of GABA(A) receptors, *J Neurosci*, 28 (2008) 1557–1567. [PubMed: 18272677]
- [10]. Rivera C, Voipio J, Thomas-Crusells J, Li H, Zsuzsa H, Sipila S, Payne JA, Minichiello L, Saarma M, Kaila K, Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter KCC2, *J Neurosci*, 24 (2004) 4683–4691. [PubMed: 15140939]
- [11]. Kaila K, Ruusuvuori E, Voipio J, Puskarjov M, GABA actions and ionic plasticity in epilepsy, *Curr Opin Neurobiol*, 26 (2014) 34–41. [PubMed: 24650502]
- [12]. Ben-Ari Y, Khalilov I, Kahle KT, Cherubini E, The GABA excitatory/inhibitory shift in brain maturation and neurological disorders, *Neuroscientist*, 18 (2012).
- [13]. Jantzie LL, Getsy PM, Firl DJ, Wilson CG, Miller RH, Robinson S, Erythropoietin attenuates loss of potassium chloride co-transporters following prenatal brain injury., *Molecular and cellular neurosciences*, 61 (2014) 152–162. [PubMed: 24983520]
- [14]. Sivakumaran S, Cardarelli RA, Maguire J, Kelley MR, Silayeva L, Morrow DH, Mukherjee J, Moore YE, Mather RJ, Duggan ME, Brandon NJ, Dunlop J, Zicha S, Moss SJ, Deeb TZ, Selective inhibition of KCC2 leads to hyperexcitability and epileptiform discharges in hippocampal slices and in vivo, *J Neurosci*, 35 (2015) 8291–8296. [PubMed: 26019342]
- [15]. Chen L, Levesque M, Avoli M, KCC2 antagonism increases neuronal network excitability but disrupts ictogenesis in vitro, *J Neurophysiol*, 122 (2019) 1163–1173. [PubMed: 31339790]
- [16]. Ronen GM, Buckley D, Penney S, Streiner DL, Long-term prognosis in children with neonatal seizures: a population-based study, *Neurology*, 69 (2007) 1816–1822. [PubMed: 17984448]
- [17]. Hill CA, Fitch RH, Sex differences in mechanisms and outcome of neonatal hypoxia-ischemia in rodent models: implications for sex-specific neuroprotection in clinical neonatal practice, *Neurology research international*, 2012 (2012) 867531. [PubMed: 22474588]
- [18]. Nunemaker CS, Tony DeFazio RA, Moenter SM, A targeted extracellular approach for recording long-term firing patterns of excitable cells: A practical guide, *Biol Proceed Online*, 5 (2003) 52–62.
- [19]. Sampath D, White AM, Raol YH, Characterization of neonatal seizures in an animal model of hypoxic-ischemic encephalopathy, *Epilepsia*, 55 (2014) 985–993. [PubMed: 24836645]
- [20]. Delpire E, Baranczak A, Waterson AG, Kim K, Kett N, Morrison RD, Daniels JS, Weaver CD, Lindsley CW, Further optimization of the K-Cl cotransporter KCC2 antagonist ML077: development of a highly selective and more potent in vitro probe, *Bioorganic & medicinal chemistry letters*, 22 (2012) 4532–4535. [PubMed: 22727639]
- [21]. Sedmak G, Jovanov-Milosevic N, Puskarjov M, Ulamec M, Kruslin B, Kaila K, Judas M, Developmental Expression Patterns of KCC2 and Functionally Associated Molecules in the Human Brain, *Cereb Cortex*, 26 (2016) 4574–4589. [PubMed: 26428952]
- [22]. Blaesse P, Airaksinen MS, Rivera C, Kaila K, Cation-chloride cotransporters and neuronal function, *Neuron*, 61 (2009).
- [23]. Stein V, Hermans-Borgmeyer I, Jentsch TJ, Hubner CA, Expression of the KCl cotransporter KCC2 parallels neuronal maturation and the emergence of low intracellular chloride, *J Comp Neurol*, 468 (2004) 57–64. [PubMed: 14648690]
- [24]. Khalilov I, Chazal G, Chudotvorova I, Pellegrino C, Corby S, Ferrand N, Gubkina O, Nardou R, Tyzio R, Yamamoto S, Jentsch TJ, Hubner CA, Gaiarsa JL, Ben-Ari Y, Medina I, Enhanced synaptic activity and epileptiform events in the embryonic KCC2 deficient hippocampus, *Frontiers in cellular neuroscience*, 5 (2011) 23. [PubMed: 22065950]
- [25]. Chamma I, Chevy Q, Poncer JC, Levi S, Role of the neuronal K-Cl co-transporter KCC2 in inhibitory and excitatory neurotransmission, *Frontiers in cellular neuroscience*, 6 (2012) 5. [PubMed: 22363264]

- [26]. Tillman L, Zhang J, Crossing the chloride channel: The current and potential therapeutic value of the neuronal K⁺-Cl⁻ cotransporter KCC2, *Biomed Res Int*, 2019 (2019) 1–12.
- [27]. Robinson S, Mikolaenko I, Thompson I, Cohen ML, Goyal M, Loss of cation-chloride cotransporter expression in preterm infants with white matter lesions: Implications for the pathogenesis of epilepsy, *J Neuropathol Exp Neurol*, 69 (2010) 565–572. [PubMed: 20467335]
- [28]. Cleary RT, Sun H, Huynh T, Manning SM, Li Y, Rotenberg A, Talos DM, Kahle KT, Jackson M, Rakhade SN, Berry G, Jensen FE, Bumetanide enhances phenobarbital efficacy in a rat model of hypoxic neonatal seizures, *PLoS One*, 8 (2013) e57148. [PubMed: 23536761]
- [29]. Ma J, Zhang S, Guo L, Li Y, Li Q, Wang S, Liu H, Wang C, KCC2 expression changes in diazepam-treated neonatal rats with hypoxia-ischemia brain damage, *Brain Res*, 1563 (2014) 22–30. [PubMed: 24709118]
- [30]. Carter BM, Sullivan BJ, Landers JR, Kadam SD, Dose-dependent reversal of KCC2 hypofunction and phenobarbital-resistant neonatal seizures by ANA12, *Sci Rep*, 8 (2018) 11987. [PubMed: 30097625]
- [31]. Dzhala V, Ben-Ari Y, Khazipov R, Seizures accelerate anoxia-induced neuronal death in the neonatal rat hippocampus, *Ann Neurol*, 48 (2000) 632–640. [PubMed: 11026447]
- [32]. Glass HC, Glidden D, Jeremy RJ, Barkovich AJ, Ferriero DM, Miller SP, Clinical Neonatal Seizures are Independently Associated with Outcome in Infants at Risk for Hypoxic-Ischemic Brain Injury, *J Pediatr*, 155 (2009) 318–323. [PubMed: 19540512]
- [33]. Pressler R, Boylan GB, Marlow N, Blennow M, Chiron C, Cross JH, de Vries LS, Halberg B, Hellstrom-Westas L, Jullien V, Mangum B, Murray D, Pons G, Rennie J, Swarte R, Toet MC, Vanhatalo S, Zohar S, Bumetanide for the treatment of seizures in newborn babies with hypoxic ischaemic encephalopathy (NEMO): an open-label, dose finding, and feasibility phase 1/2 trial, *Lancet*, 14 (2015) 469–477. [PubMed: 25765333]

Highlights

- The functional role of KCC2 in preventing overexcitation in neonatal brain is not clear
- We show that reducing KCC2 activity exacerbates neonatal brain excitability
- KCC2 is a potential therapeutic target to reduce brain injury in newborns

Figure 1. KCC2 inhibitor, VU0463271, increases hippocampal pyramidal cell excitability. A representative trace shows spiking activity (A) before (baseline) and after (B) VU0463271 (VU) application in hippocampal slices prepared from P7-P9 male rat pups. (C) Bath application of VU significantly increased spiking frequency of CA1 hippocampal neurons. The bar graph shows the difference in the spiking frequency that occurred before and after the drug application. * $p < 0.05$, $n = 8$ neurons in each group.

Figure 2. Representative EEG tracings from either vehicle or VU0463271 treated P7 rats. An example of contralateral (to ligated carotid artery) cortex (C-Cx) and ipsilateral cortex (I-Cx) EEG acquired from a rat pup before HI-induction and treatment with either (A) vehicle or (B) VU0463271 (VU). An EEG trace showing spike and sharp-wave activity during hypoxia after the 2nd dose of vehicle (C) or VU (D). An EEG trace showing a seizure epoch (beginning of the seizure is marked by an arrow in C in Cx channel) in the same VU treated rat (F) and (E) an epoch at an equivalent time period from vehicle treated rat. The seizure on the EEG was behaviorally associated with clonic movements of limbs. S = second, μV = microvolts.

Figure 3. Inhibition of KCC2 activity increases ictal activity under hypoxic conditions.

(A) Significantly more ictal events (seizures + BREDs combined) were observed during hypoxic period in rats that were administered VU0463271 (VU) compared to vehicle injected rats. Individually, on average, more seizures (B) and BREDs (C) were observed in rats that were given VU than the vehicle treated rats, however, the difference did not reach statistical significance. Administration of VU did not affect duration of either ictal (D) or seizure (E) events, but it increased average duration of BREDs (F). Ictal events shown in the figure include both full-blown seizures (EEG seizures with a behavioral correlate lasting 10 seconds or more) and brief runs of epileptiform discharges (BREDs) characterized by EEG patterns that were associated with a behavioral correlate that lasted 3–9 seconds. * $p < 0.05$, $n = 5$ pups in each group.

Figure 4. Inhibition of KCC2 activity increases *in vivo* spike and sharp-wave activity under hypoxic conditions.

A significant increase in spike and sharp-wave activity during the inter-ictal period was observed in rats that were administered VU0463271 (VU) when compared with the vehicle injected rats under hypoxic conditions. Ten-minutes-long epochs of EEG recordings during baseline and 10 minutes after the 1st and 2nd dose of drug administration (VU or vehicle) were manually scanned to determine the number of spikes and sharp-waves. The number of spikes and sharp-waves observed in the baseline record were deducted from that observed during hypoxia to obtain the final count presented in the figure. ** $p < 0.01$, $n = 5$ pups in each group.

Figure 5. *In vivo* levels of VU0463271 detected in brain tissues and serum.

(A) A representative LC-MS/MS chromatogram from a neonatal rat hippocampus. (B) A histogram showing concentration of VU0463271 (VU) in various brain regions and serum. Cortical tissue was obtained from sensory and motor cortices. Data presented is mean $\pm$ SD for five animals. HP = Hippocampus, Re Brain = Remaining brain tissue.