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Reducing premature KCC2 expression rescues seizure susceptibility and spine morphology in atypical febrile seizures

Patricia N. Awad [a,b], Nathalie T. Sanon [b], Bidisha Chattopadhyaya [b], Josianne Nunes Carriço [b], Mohamed Ouardouz [b], Jonathan Gagné [a,b], Sandra Duss [b], Daniele Wolf [a,b], Sébastien Desgent [b], Laura Cancedda [c], Lionel Carmant [a,b,*], Graziella Di Cristo [a,b,*]

[a] Neurosciences Department, Université de Montréal, 2960 Chemin de la Tour, Montréal, Québec H3T 1N8, Canada
[b] CHU Sainte-Justine Research Center, 3175 Chemin de la Côte Sainte-Catherine, Montréal, Québec H3T 1C5, Canada
[c] Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, via Morego, 30, 16163 Genova, Italy

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ABSTRACT

Atypical febrile seizures are considered a risk factor for epilepsy onset and cognitive impairments later in life. Patients with temporal lobe epilepsy and a history of atypical febrile seizures often carry a cortical malformation. This association has led to the hypothesis that the presence of a cortical dysplasia exacerbates febrile seizures in infancy, in turn increasing the risk for neurological sequelae. The mechanisms linking these events are currently poorly understood. Potassium-chloride cotransporter KCC2 affects several aspects of neuronal circuit development and function, by modulating GABAergic transmission and excitatory synapse formation. Recent data suggest that KCC2 downregulation contributes to seizure generation in the epileptic adult brain, but its role in the developing brain is still controversial.

In a rodent model of atypical febrile seizures, combining a cortical dysplasia and hyperthermia-induced seizures (LHS rats), we found a premature and sustained increase in KCC2 protein levels, accompanied by a negative shift of the reversal potential of GABA. In parallel, we observed a significant reduction in dendritic spine size and mEPSC amplitude in CA1 pyramidal neurons, accompanied by spatial memory deficits. To investigate whether KCC2 premature overexpression plays a role in seizure susceptibility and synaptic alterations, we reduced KCC2 expression selectively in hippocampal pyramidal neurons by *in utero* electroporation of shRNA. Remarkably, KCC2 shRNA-electroporated LHS rats show reduced hyperthermia-induced seizure susceptibility, while dendritic spine size deficits were rescued. Our findings demonstrate that KCC2 overexpression in a compromised developing brain increases febrile seizure susceptibility and contribute to dendritic spine alterations.

1. Introduction

Febrile seizures (FSs) are the most common convulsive events in children between 6 months and 5 years of age, with a prevalence of 2–14% in this population. Simple FSs are considered benign, whereas atypical febrile seizures, which account for 30–40% of FSs cases (Nelson and Ellenberg, 1976), are a risk factor for Mesial Temporal Lobe Epilepsy (MTLE) (Finegersh et al., 2011; French et al., 1993; Hamati-Haddad and Abou-Khalil, 1998). In fact, while only 2% of all children with FSs will develop epilepsy, the long-term risk of developing MTLE is much higher (32–49%, depending on the cohorts) for children with FSs that have all three atypical features: lateralized, prolonged and multiple FSs in a day (Annegers et al., 1987; Birca et al., 2004). Notably, individuals carrying a developmental defect in cortical architecture, termed focal cortical dysplasia, are more likely to develop atypical febrile seizures than the general population (Bocti et al., 2003; Hesdorffer et al., 2008). Studies in animal models as well as in patients with MTLE support a causal link between the presence of cortical dysplasia, the incidence of atypical FSs, and the subsequent development of hippocampal sclerosis and MTLE (Park et al., 2010; Scantlebury et al., 2004; Tassi et al., 2010; Tassi et al., 2009). Based on these observations, we developed a rodent model combining both focal cortical dysplasia and FSs (LHS rats). Pups carrying a cortical dysplasia show shorter seizure onset time and more prolonged seizures when exposed to hyperthermia at P10 compared to naïve rats (Scantlebury et al., 2004), a condition resembling febrile status epilepticus in children. In addition, by adulthood, most LHS rats develop spontaneous recurrent seizures and hippocampal sclerosis accompanied by synaptic alterations and spatial memory deficits (Gibbs et al., 2011; Scantlebury et al., 2005). Thus, this model is clinically relevant, as it reproduces well the

* Corresponding authors at: Research Center, CHU Ste-Justine/Université de Montréal, 3175, Côte-Sainte-Catherine, Montréal, QC H3T 1C5, Canada.
E-mail addresses: lionel.carmant@umontreal.ca (L. Carmant), graziella.dicristo@recherche-ste-justine.qc.ca (G. Di Cristo).
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association between the presence of a predisposing brain malformation, atypical FSs and the development of neurological sequelae. The mechanisms underlying increased atypical FSs susceptibility in brains carrying a focal dysplasia are poorly understood.

KCC2 and NKCC1 are cation-chloride cotransporters, which are responsible for establishing the neuronal Chloride (Cl^-) gradient that governs GABAergic inhibition. During development, the increase in KCC2 expression is responsible for the shift of GABA function from excitatory to shunting/inhibitory (Kaila et al., 2014). In addition to regulating the Cl^- gradient, KCC2 modulates several aspects of neuronal development, including synapse formation (Fiumelli et al., 2013; Gauvain et al., 2011; Li et al., 2007). The immaturity of the GABAergic system and the presence of higher levels of intracellular Cl^- in the developing neurons may in part account for the higher incidence of seizures during early postnatal development (Dzhala et al., 2005). This hypothesis led to the prediction that KCC2-activating or NKCC1-inibiting compounds would improve seizure controls in neonates (Dzhala et al., 2005); however clinical trials testing this hypothesis have been so far disappointing (Pressler et al., 2015). Recent data suggest that different traumatic events in the developing brain, including seizures, induce an increase in KCC2 expression levels and/or activation (Galanopoulou, 2008; Khirug et al., 2010; Puskarjov et al., 2014). Thus, KCC2/NKCC1 balance may be altered in a brain with pre-existing conditions, making it harder to predict the outcome of targeting their activity on seizure susceptibility. Further, whether and to what extent pathology-induced premature KCC2 expression contributes to long-term synaptic alterations remains to be investigated.

Here, we test the hypothesis that KCC2 expression and function is prematurely increased in the LHS model, and that this event causes increased seizure susceptibility and altered synapse formation in the developing brain.

2. Materials and methods

2.1. Animals

Sprague–Dawley pups at postnatal day 1 (P1) or pregnant females (embryos at embryonic day 10 (E10) were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). Pups were culled to 12 per dam, matched by gender, weighed and kept with their mother in a 12 h light/dark cycle with food and water *ad libitum*. Animal care and use conformed to institutional policies and guidelines (CIBPAR, Sainte-Justine Hospital Research Centre, Université de Montréal, Montreal, QC, Canada). This study complies with the ARRIVE guidelines.

2.2. Freeze lesions and induction of hyperthermic seizures

A focal microgyrus and hyperthermic seizures were induced as described in Scantlebury et al. (2004). Briefly, in anesthetized P1 rats, a cylindrical 2-mm diameter copper probe, cooled in liquid nitrogen, was brought into contact with the skull overlying the right fronto-parietal cortex, 2 mm caudal to bregma and 2 mm lateral to the sagittal suture, for 10 s after skin incision. At P10, pups were placed individually at the bottom of a Plexiglas box through which warm dry air (45–52 °C) was circulated by a standard hairdryer fitted on the uppermost part of a side panel. Each pup remained in the box until a generalized convulsion occurred. Pups were then moved to an ambient temperature surface and remained untouched for 30 min of observation.

2.3. Western blot

Western blots were performed as described in Ouardouz et al. (2010). Membranes were probed with the following primary antibodies: anti-KCC2 1:1000 (rabbit polyclonal IgG; Cat. no. 07-432, Millipore) and 1:200 anti-NKCC1 (kindly gifted by Dr. Jim Turner, NIH/NIDCR) and anti-glyceraldehyde-3-phosphate dehydrogenase 1:4000 (GAPDH, mouse monoclonal IgG; Cat. no. AM4300; Applied Biosystems, Streetsville, Ontario, Canada). Each experimental group included 3 to 5 animals. All samples were run simultaneously. Bands were quantified using ImageJ software. The intensity of each KCC2 and NKCC1 band was first normalized over the intensity of the GAPDH band in the same lane (internal loading control). For each experimental group, KCC2 intensity levels were then normalized over the control group represented by naïve animals of the same sex.

2.4. Electrophysiology

Electrophysiological recordings were essentially performed as in Ouardouz et al. (2010). Hippocampal slices were prepared from male rats at P18–P22 (mEPSC experiment) or P11–P15 (E_{GABA} experiment). Male rats were anesthetized with isoflurane and decapitated. Brain tissue was quickly removed and placed in cold artificial cerebrospinal fluid (ACSF) containing in mM: 126 NaCl, 3 KCl, 2 $MgSO_4$–H_2O, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 D-Glucose, bubbled with 95% O_2/− 5% CO_2. The pH and osmolarity were adjusted to 7.3–7.4 and 300–310 mOsm, respectively. Hippocampal slices (300 μm thick) were cut with a vibratome (VT1000S, Leica Microsystems) and transferred to a container filled with oxygenated ACSF at room temperature. After an hour of incubation, individual slices were placed into a recording chamber and continuously superfused with oxygenated ACSF heated at ±32 °C with a temperature controller (TC-324B, Warner Instruments).

Hippocampal CA1 pyramidal cells were visualized with an upright microscope (Olympus) fitted with a near-infrared CCD camera (XC-EI50, Sony). Patch pipettes were pulled from borosilicate glass tubing with a resistance ranging from 4 to 7 MΩ. Intracellular solutions used were K^+-based or Cs^+-based according to the experiment and contained in mM: 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 Hepes buffer, 0.5 EGTA, 10 phosphocreatine, 2 ATP Tris, 0.4 GTP Li. For CsCl-based solution all K^+ ions were replaced by Cs^+, and the solution included the Na^+ inhibitor N-(2,6-dimethyl-phenylcarbamoylmethyl) triethylammonium bromide QX-314 (2 mM; Sigma). The pH was adjusted to 7.2–7.3 with KOH or CsOH, respectively, and biocytin was added to the intracellular solutions for post-hoc confirmation of cell identity. Signals were digitized with a Digidata 1440 A analog-digital converter (Molecular Devices), acquired at a sampling rate of 2 kHz, low-pass filtered at 1 kHz using an Axopatch 200B amplifier (Axon Instruments) and visualized using pClamp software 10 (Molecular Devices). Miniature excitatory postsynaptic currents (mEPSCs) were recorded in whole-cell configuration at a holding potential of −60 mV and in presence of $GABA_A$ receptor antagonist Bicuculline methiodide (5 μM) and TTX (1 μM). All events detected over 10 min were analyzed. A total of 11 animals (11 cells from 6 male LHS, 10 cells from 5 male Ctrl) were used for these experiments. To measure the reversal potential of GABA (E_{GABA}), eIPSCs were evoked by a puff of 10 μM GABA delivered in the vicinity of the CA1 pyramidal cell soma (30 psi, 3 ms, 20 s interval). In this protocol, 7.5 μg/ml of gramicidin diluted in DMSO, and Alexa Fluor 488 were added to the intracellular solution containing in mM: 135 KCl, 4 NaCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES. E_{GABA} current amplitudes were measured at the peak current evoked at 10 mV incrementing holding potentials from −120 to −30 mV, E_{GABA} value representing the intercept of the current–voltage curve with the abscissa. All drugs were purchased from Sigma-Aldrich (Canada). A total of 13 male rats (7 cells from 6 LHS, 8 cells from 7 Ctrl) were used for these experiments.

2.5. DiI labeling

DiI labeling was performed essentially as previously described in Gibbs et al. (2011). Labeled pyramidal cells were imaged 40–60 min after diolistic transfection. Only pyramidal cells with a complete soma and clearly labeled primary basal dendrite branches were imaged and traced. Because of the variability of DiI labeling, we chose to reconstruct