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Research Paper

Locally Reducing KCC2 Activity in the Hippocampus is Sufficient to Induce Temporal Lobe Epilepsy

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ABSTRACT

Mesial temporal lobe epilepsy (mTLE) is the most common form of epilepsy, believed to arise in part from compromised GABAergic inhibition. The neuronal specific K^+/Cl^- co-transporter 2 (KCC2) is a critical determinant of the efficacy of GABAergic inhibition and deficits in its activity are observed in mTLE patients and animal models of epilepsy. To test if reductions of KCC2 activity directly contribute to the pathophysiology of mTLE, we locally ablated KCC2 expression in a subset of principal neurons within the adult hippocampus. Deletion of KCC2 resulted in compromised GABAergic inhibition and the development of spontaneous, recurrent generalized seizures. Moreover, local ablation of KCC2 activity resulted in hippocampal sclerosis, a key pathological change seen in mTLE. Collectively, our results demonstrate that local deficits in KCC2 activity within the hippocampus are sufficient to precipitate mTLE.

1. Introduction

Mesial temporal lobe epilepsy (mTLE) is the most common form of human epilepsy, classified by unprovoked, spontaneous seizures originating within the temporal lobe. mTLE in humans develops over a latent period of several years after a precipitating insult such as traumatic brain injury, hypoxia, infection, or episodes of status epilepticus [1,2]. In addition to seizures, profound morphological changes are observed in the brain, the most common being hippocampal sclerosis [3]. Sclerotic hippocampal tissue surgically removed from human mTLE patients shows widespread neuronal loss and gliosis [4]. Similar changes are seen in convulsant-induced rodent models of mTLE [5].

While the cellular mechanisms that underlie the development of mTLE remain poorly understood, deficits in the efficacy of γ-aminobutyric acid (GABA) mediated inhibitory neurotransmission are widely believed to be of significance [6–8]. Fast synaptic inhibitory transmission in the brain is primarily mediated by chloride (Cl^-)-permeable γ-aminobutyric acid receptors ($GABA_A$Rs), which upon opening hyperpolarize the cell membrane through Cl^- influx and decrease neuronal firing. During development, the electroneutral K^+/Cl^- co-transporter 2 (KCC2) becomes the dominant mediator of Cl^- extrusion, coupling Cl^- efflux to the outwardly directed K^+ gradient [9,10]. Deficits in KCC2-mediated Cl^- extrusion result in increased intracellular Cl^- levels, permitting depolarizing $GABA_A$R responses [11]. KCC2 loss-of-function mutations in human patients result in severe cases of epilepsy in infancy with migrating focal seizures [12,13]. In mice, a global loss of 95% of KCC2 results in seizures and mortality at 2–3 weeks postnatal [14], and loss-of-function mutations increase seizure susceptibility [15,16].

Reduced KCC2 levels and depolarizing $GABA_A$R signaling are observed in tissue surgically removed from mTLE patients [17–20]. In rodent models of mTLE, reduced KCC2 is observed in the hippocampus immediately after an initial precipitating injury and during subsequent epileptogenesis [21–25]. However, it is unknown if these deficits directly contribute to the development of seizures and accompanying pathophysiology of mTLE.

To test this, we have ablated KCC2 expression in a subset of principal neurons in the hippocampus of adult mice. We show that reducing KCC2 activity leads to increased neuronal Cl^- accumulation and gross deficits in GABAergic inhibition. Moreover, these deficits are sufficient to initiate recurring generalized seizures, reactive astrogliosis, and neuronal loss within the hippocampus, reproducing the core features of mTLE with hippocampal sclerosis. Thus, local inhibition of KCC2 within the hippocampus is sufficient to precipitate mTLE.

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2. Materials and Methods

2.1. Animal Care

Animal studies were performed with protocols approved by the Institutional Animal Care and Use Committee of Tufts Medical Center. Mice were kept on a 12-h light/dark cycle with *ad libitum* access to food and water.

2.2. Generation and Genotyping of $KCC2^{FL}$ Mice

$KCC2^{FL}$ mice (*Slc12a5*$^{lox/lox}$) were have been described previous and have been backcrossed on the C57BL/6 J mice for 10 generations [26]. PCR was with an expected product size of 426 bp for wild-type and 543 bp for $KCC2^{FL}$ mice.

2.3. Viral Injections

Adult (6–8 week old) male $KCC2^{FL}$ mice were stereotaxically injected with adeno-associated virus (AAV) containing *Cre* recombinase. Prior to surgery, mice were injected with buprenorphine (0.75 mg/kg, s.c.) and anesthetized with isoflurane (1–3%). Drill holes were made above each dorsal hippocampus (coordinates: AP -2.0 mm from bregma, ML +/− 1.8 mm) and a Neuros syringe (Hamilton, Reno, NV) lowered to 2.0 mm below the brain surface. Mice were bilaterally injected with 0.5 μL of either AAV9·CaMKII·HI.eGFP-*Cre*.WPRE.SV40 or AAV9·CaMKII0.4.eGFP·WPRE.rBG (titer: 1×10^{13} GC/mL in PBS, University of Pennsylvania Vector Core, Philadelphia, PA) at a rate of 50 nL/min. After injection, the needle remained in the brain 5 min before being withdrawn. The skin was closed and mice placed on a heating pad until mobile. Mice were singly housed post-surgery.

2.4. EEG/EMG and Video Recordings

A subset of virus-injected mice were implanted with electroencephalography/electromyography (EEG/EMG) headstages (2-channel, Pinnacle Technology, Lawrence, KA) following virus injection. Implants were aligned with lambda and 4 screws inserted for subdural recording contacts above the right frontal and parietal lobes. The implant was glued to the skull and mice recovered as above. After a recovery period of 7 days, continuous paired video and EEG/EMG recordings were collected using Sirenia Acquisition software (Pinnacle). Recordings were processed using Sirenia Seizure (Pinnacle) and EEG seizure events detected using DClamp (Massachusetts General Hospital Pediatric Epilepsy Research Lab, Boston, MA). Seizure FFT spectra were created using MATLAB (Mathworks, Natick, MA).

2.5. Slice Immunohistochemistry

Mouse brains were fixed by transcardial perfusion of PBS-buffered 4% paraformaldehyde (PFA) (Electron Microscopy Services, Hatfield, PA) and removed to 4% PFA overnight, then cryoprotected in 30% sucrose solution in PBS for 3 days before being frozen in optimal cutting temperature medium (VWR, Radnor, PA). 40 μm serial coronal sections were prepared using a Leica SM-2000R microtome (Wetzlar, Germany). Sections were kept at −20°C in cryoprotectant solution (876 mM sucrose, 4 M polyvinylpyrrolidone, 30% ethylene glycol, and 10% PBS in diH_2O), and washed with PBS before blocking. Sections were blocked with 10% normal goat serum (NGS) and 0.3% Triton X-100 in PBS for 2 h at room temperature. Sections were incubated overnight with either anti-KCC2 C-terminus (1:500 rabbit, Millipore 07–432, Darmstadt, Germany), anti-GFAP (1:5000 mouse, Millipore MAB360), or anti-NeuN (1:1000 mouse, Millipore MAB377). Slices were subsequently labeled with Alexa-Fluor secondary antibodies (1:200; Thermo Fisher Scientific, Waltham, MA) prior to mounting with ProLong Gold with or without DAPI (Thermo Fisher). To measure KCC2 expression levels *Z*-stack images were taken of the hippocampus at 20× using an Eclipse Ti confocal microscope (Nikon, Tokyo, Japan). Regions of interest (ROI) were drawn in the CA1 or DG and the average intensity for each ROI was normalized to the maximum average intensity per animal. Slices of each animal were averaged and compared between groups at equivalent time-points after virus injection. For NeuN, images were taken at 60× of CA1 principal layer or dentate gyrus granule cell layer, and 100 μm^2 ROIs drawn. NeuN positive labeled cells were counted in each ROI, averaged for each animal and compared between groups. For GFAP labeling, images were obtained using a scanning fluorescence microscope (BZ-X700, Keyence, Osaka, Japan) at 10x magnification. ROIs were drawn around the CA1 or DG in each slice. A threshold was used create a binary mask of GFAP labeling, and area calculated for ROI and reported as amount of GFAP labeling/mm^2. Values from each animal were averaged and then compared between groups. All image processing was performed using FIJI (ImageJ).

2.6. Slice Electrophysiology

Mice were anesthetized with isofluorane and brains removed. Coronal slices (350 μm) were cut on a Leica VT1000s vibratome in ice-cold cutting solution containing (in mM): 87 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 7 $MgCl_2$, 50 sucrose and 25 glucose, pH 7.4. Slices were placed in a submerged chamber for a 1 h recovery period at 32 °C in artificial cerebrospinal fluid (aCSF) containing (in mM): 126 NaCl, 26 $NaHCO_3$, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1 glutamine, 1.25 NaH_2PO_4, 1.5 Na-pyruvate and 10 glucose. All solutions were bubbled with 95% O_2/5% CO_2. *Gramicidin Perforated Patch Recordings.* After a 1-h recovery, slices were placed in a submerged chamber (RC-27 L, Warner Instruments, Hamden, CT). For perforated patch, micropipettes (3–4 MΩ) contained saline (in mM): 140 KCl and 10 HEPES, pH 7.4 KOH, and were backfilled with gramicidin D (50 μg/mL, Sigma Aldrich) to establish access resistances between 20 and 80 MΩ throughout the recording period. Dentate gyrus granule cells were patched in the outer 2/3 of the granule cell layer to avoid newborn granule cells. A glass pipette (1–3 MΩ tip resistance) filled with muscimol (10 μM, Tocris Bioscience, Bristol, UK) in aCSF was lowered into the molecular layer of the dentate gyrus and pulses (500 ms) applied locally by pressure ejection with a Picospritzer II (General Valve, Fairfield, NJ) to activate $GABA_A$-mediated currents in granule cells held at voltages between −100 to −50 mV in 10-mV increments. E_{GABA} values were obtained from linear regression fits to the data at voltages near the observable reversal potential of $I_{muscimol}$. Tetrodotoxin (TTX, 400 nM, Tocris) was applied to block activity-dependent shifts in E_{GABA} and bumetanide (10 μM, Tocris) to inhibit NKCC1, and VU0463271 to inhibit KCC2 (1 μM, AstraZeneca, Cambridge, UK). Voltages were corrected offline with a liquid junction potential value of 3.8 mV. Recordings were performed with a Multiclamp 700B amplifier and Clampex 10 acquisition software. Data were low-pass filtered at 10 kHz and analyzed offline with Clampfit (Molecular Devices, Sunnyvale, CA).

2.7. Primary Neuron Culture

Primary cortical/hippocampal neurons were prepared and cultured as previously described [27]. Briefly, P0 $KCC2^{FL}$ mice were anesthetized on ice and the brains removed. The brains were dissected in Hank's buffered salt solution (HBSS, Invitrogen) with 10 mM HEPES. The cortices and hippocampi were tripsinized and triturated to dissociate the neurons. Cells were counted using a hemocytometer and plated on poly-L-lysine-coated coverslips (for ICC and electrophysiology) or in 35 mm dishes (for immunoblot) at a density of 1×10^5 or 4×10^5 cells respectively. At days *in vitro* (DIV) 18, cells were exposed to control or *Cre* AAV as described above at a concentration of 1×10^6 GC/mL. To determine effect on neuronal viability, GFP-positive cells were counted at DIV 24.

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