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HOMEOSTATIC REGULATION OF KCC2 ACTIVITY BY THE ZINC RECEPTOR mZnR/GPR39 DURING SEIZURES

David Gilad[1], **Sharon Shorer**[1], **Maya Ketzef**[1], **Alon Friedman**[1], **Israel Sekler**[1], **Elias Aizenman**[1,2], and **Michal Hershfinkel**[1,*]

[1]Department of Physiology and Cell Biology, Ben-Gurion University of the Negev, Faculty of Health Sciences, Beer-Sheva, Israel

[2]Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Abstract

The aim of this study was to investigate the role of the synaptic metabotropic zinc receptor mZnR/GPR39 in physiological adaptation to epileptic seizures. We previously demonstrated that synaptic activation of mZnR/GPR39 enhances inhibitory drive in the hippocampus by upregulating neuronal K^+/Cl^- co-transporter 2 (KCC2) activity. Here, we first show that mZnR/GPR39 knockout (KO) adult mice have dramatically enhanced susceptibility to seizures triggered by a single intraperitoneal injection of kainic acid, when compared to wild type (WT) littermates. Kainate also substantially enhances seizure-associated gamma oscillatory activity in juvenile mZnR/GPR39 KO hippocampal slices, a phenomenon that can be reproduced in WT tissue by extracellular Zn^{2+} chelation. Importantly, kainate-induced synaptic Zn^{2+} release enhances surface expression and transport activity of KCC2 in WT, but not mZnR/GPR39 KO hippocampal neurons. Kainate-dependent upregulation of KCC2 requires mZnR/GPR39 activation of the Gαq/phospholipase C/extracellular regulated kinase (ERK1/2) signaling cascade. We suggest that mZnR/GPR39-dependent upregulation of KCC2 activity provides homeostatic adaptation to an excitotoxic stimulus by increasing inhibition. As such, mZnR/GPR39 may provide a novel pharmacological target for dampening epileptic seizure activity.

Keywords

Epilepsy; seizure; hippocampus; zinc receptor; mZnR/GPR39; KCC2; kainic acid

Introduction

Zn^{2+} is the second most prevalent trace element in the human body, distributed widely throughout the brain (Frederickson *et al.* 2005; Sensi *et al.* 2011) and selectively stored within presynaptic vesicles in a sub-population of excitatory neurons by the neuronal-

*Corresponding author at: Department of Physiology and Cell Biology, Ben-Gurion University of the Negev, Faculty of Health Sciences, Beer-Sheva, 84015 Israel. Phone: +972-864-77318, hmichal@bgu.ac.il.

specific zinc transporter ZnT3 (Cole *et al.* 1999; Cole *et al.* 2000; Sensi *et al.* 2011). Vesicular Zn^{2+} is co-released with glutamate into the synaptic cleft in a Ca^{2+}- and activity-dependent manner (Li *et al.* 2001; Qian *et al.* 2005; Paoletti *et al.* 2008; Perez-Rosello *et al.* 2013; Vergnano *et al.* 2014). Synaptically-released Zn^{2+} has been suggested to regulate neuronal excitability and modulate the severity of seizure activity (Vogt *et al.* 2000; Smart *et al.* 2004; Sensi *et al.* 2009). Indeed, genetic removal of synaptic Zn^{2+} via ZnT3 deletion leads to enhanced susceptibility to epileptic seizures (Cole *et al.* 1999; Cole *et al.* 2000), and importantly, deficiencies in plasma Zn^{2+} levels have been associated with human epileptic disorders (Goldberg *et al.* 1982; Blasco-Ibanez *et al.* 2004; Ganesh *et al.* 2008; Farahani *et al.* 2013; Seven *et al.* 2013; Wojciak *et al.* 2013; Saad *et al.* 2014). Moreover, studies in kindling models of epilepsy suggest that seizure activity can be moderated via Zn^{2+} administration (Elsas *et al.* 2009; Baraka *et al.* 2012). Nonetheless, the pathways linking synaptically released Zn^{2+} to regulation of seizure activity are poorly understood.

The metabotropic Zn^{2+}-sensing receptor (mZnR/GPR39) has previously been suggested to be present in the soma and dendrites of pyramidal neurons in the CA3 region of the hippocampus (Jackson *et al.* 2006; Besser *et al.* 2009). This receptor is activated by synaptic Zn^{2+} released from the mossy fibers and triggers an intracellular Ca^{2+} rise in hippocampal CA3 neurons, but not glia (Besser *et al.* 2009). Activation of mZnR/GPR39 signaling leads to upregulation of K^+/Cl^- cotransporter 2 (KCC2) surface expression and activity (Chorin *et al.* 2011; Saadi *et al.* 2012). As KCC2 is the major Cl^- extruding transporter in neurons, responsible for hyperpolarizing currents mediated by $GABA_A$ receptor channel activation (Rivera *et al.* 1999; Zhu *et al.* 2005; Viitanen *et al.* 2010), a hyperpolarizing shift in the GABA reversal potential was measured in pyramidal CA3 neurons following exposure to Zn^{2+} (Chorin *et al.* 2011).

KCC2 activity is essential for regulating neuronal inhibitory drive (Blaesse *et al.* 2009). KCC2 expression, induced during the first two postnatal weeks (Rivera *et al.* 1999; Ludwig *et al.* 2003; Stein *et al.* 2004), correlates with the developmental switch from depolarizing to hyperpolarizing $GABA_A$ receptor-mediated synaptic potentials (Rivera *et al.* 2002; Rivera *et al.* 2004; Deeb *et al.* 2011). Moreover, overexpression of KCC2 in immature neurons is sufficient to induce inhibitory GABA currents (Lee *et al.* 2005). Of note, KCC2-deficient animals die shortly after birth as a result of fulminant epileptic seizures (Woo *et al.* 2002; Zhu *et al.* 2008), and changes in KCC2 have been tightly linked to human epilepsy (Veliskova *et al.* 2004; Huberfeld *et al.* 2007; Puskarjov *et al.* 2014). The activity of KCC2 is highly regulated by changes in its surface expression and phosphorylation (Lee *et al.* 2007; Kahle *et al.* 2013), and interictal or seizure activity can induce such changes (Rivera *et al.* 2002). Notably, a single seizure episode *in-vivo* or a brief application of the excitotoxin kainic acid induce a KCC2-dependent shift in $GABA_A$ reversal potential in neonatal rat hippocampus (Khirug *et al.* 2010). Here, we show a regulatory feedback process of KCC2 activity that is triggered via Zn^{2+}-dependent mZnR/GPR39 activation during kainate-induced epileptiform activity.

Materials and methods

Mouse strains utilized

Two distinct mouse strains were utilized in this study, ICR (CD-1) outbred mice (Harlan, Israel), as well as mZnR/GPR39$^{-/-}$ (KO) and wild-type (WT) littermates, which were bred on a C57Bl6 genetic background (Moechars *et al.* 2006). The use of two mouse strains enabled us to ascertain that the putative actions of synaptic Zn^{2+} in modulating the effects of kainate were not strain specific and also allowed us to properly match experiments with KO animals to their WT genetic background. Mice were bred in a temperature-controlled room with 12-hour day/night cycles, with food and water administered ad libitum. WT and mZnR/GPR39 KO mice were genotyped using PCR of DNA isolated from tail (adult) or finger (P10) biopsy samples. Primers 5′-ACCCTCATCT TGGTGTACCT-3′ and 5′-TGTAGCGCTCAAAGCTGAG-3′ amplified a 311-bp band from the WT allele, whereas primers 5′-GGAACTCTCACTCGACCTGGG-3′ and 5′-GCAGCGC ATCGCCTTCTATC-3′ amplified a 262-bp band from the knockout allele. For *in vivo* seizure induction only mZnR/GPR39 KO and WT mice were used.

Seizure induction with kainic acid

Experimental procedures were performed in accordance with a protocol approved by the committee for the Ethical Care and Use of Animal in Experiments at the Faculty of Health Sciences at Ben-Gurion University. Adult (6 months and over) mZnR/GPR39 KO and WT mice were injected intraperitoneally with a single 10 mg/kg dose of kainic acid (KA). There were no significant differences between the average weight of WT vs. mZnR/GPR39 KO mice (28±1 g vs. 31±1 g, respectively, P=0.172). Following injections, a person blinded to the genotype observed the mice in plastic cages open to room air under quiet conditions. Behavioral seizure severity score (0-no response; 1-unresponsive; 2-focal clonic convulsion; 3-tonic/clonic seizure; 4-rearing; 5-loss of posture; 6-status epilepticus/death) (Racine 1972; Cole *et al.* 2000) was performed every 2 min for up to 1 hour. Statistical analysis was performed using Mann Whitney U-test.

Slice preparation

Hippocampal slices were obtained from postnatal day 15–20 (P15–P20) mice of either sex, following protocols aimed to preserve mossy fiber structure (Amaral *et al.* 1989; Bischofberger *et al.* 2006; Amaral *et al.* 2007). Slice experiments were performed on tissue obtained from juvenile animals in order to directly compare the electrophysiological measurements to fluorescent dye studies on the same age animals (see below). Longitudinal slices (400μm) were kept in artificial CSF (ACSF in mM: 124 NaCl, 26 NaHCO3, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 3 KCl, and 10 glucose, pH=7.4±0.1) at room temperature. To test the effects of extracellular Zn^{2+}, slices were transferred to NaH_2PO_4-free ACSF to prevent precipitation of the metal (Besser *et al.* 2009). Slices were then pretreated with kainate (300 nM, 10 min) or with vehicle alone. Following treatment, the solution was replaced with Zn^{2+}-free ACSF solution and slices were quickly placed in the recording chamber.

Electrophysiological recordings

Slices were placed in a humidified standard interface chamber at 36±1 °C under continuous ACSF perfusion (0.5–0.75 ml/min). Spontaneous activity following kainic acid (300 nM) application was recorded from the CA3 region using extracellular glass microelectrodes (1.3–1.5 MΩ, Science Products, Germany) containing (in mM): 140 NaCl, 3 KCl, 2 MgCl2, 10 glucose, 2 CaCl2, and 10 HEPES, pH 7.4. Field potentials were DC recorded and amplified (SEC-10L amplifier, NPI, Tamm, Germany), low pass filtered at 0.3 kHz, digitized on-line (POWER 1401,CED, UK) and analyzed off-line. All slices that exhibited field potential amplitudes of at least 0.5 mV in response to stimulation (0.1 Hz) with concentric tungsten electrodes (WPI, Sarasota, FL, USA), placed at the dentate gyrus, were included in the analysis.

Fluorescence imaging

For measurements of KCC2 activity, slices were loaded with BCECF AM [2′, 7′ – bis-(2-carboxyethyl)-5-(and-6-) carboxyfluorescein AM] (25 μM, TefLabs) for 15 min in the presence of 0.02% pluronic acid as described previously (Beierlein *et al.* 2002; Besser *et al.* 2009). Slices from mice at P15-P20 were used as loading of fluorescent dyes is substantially more efficient by bulk loading at this age, when compared to slices obtained from older animals, likely due to the extensive neuropil development (Peterlin *et al.* 2000). At two weeks of age, KCC2 as well as Zn^{2+} are found in the hippocampus at levels similar to those of adult neurons (Slomianka *et al.* 1997; Rivera *et al.* 1999; Nitzan *et al.* 2002; Stein *et al.* 2004), thus allowing measurements of the effects of kainate-dependent Zn^{2+} release on KCC2 activity in those tissues. Following dye loading, slices were washed for at least 30 min in ACSF. Fluorescence imaging measurements, focusing on the CA3 pyramidal cell layer subfield of the hippocampus, were acquired every 3 s (Imaging Workbench 4, INDEC BioSysPhotonics) using a 10X objective (Olympus, BX51) with 4 × 4 binning of the image (SensiCam, PCO). A bandpass emission filter of 535 nm (Chroma Technology) was utilized. R is a term representing the ratio of the emitted signal obtained using excitation wavelength of 440 nm/480 nm. For each slice, 9–15 traces from randomly selected regions of interest (ROIs), identified by a bright-field image in the pyramidal cell layer [stratum pyramidale (SP)], were acquired and averaged to give the overall rate of fluorescence change in a given slice (see Fig. 4). Bar graphs represent the average rates over several independent slices (n, values marked in figure legends). To determine the rate of KCC2 activity we applied NH_4Cl (10 mM) to the slice, which induced rapid initial alkalinization due to passive entry of NH_3 into the cells (Titz *et al.* 2006). Extracellular NH_4^+ serves as a surrogate to K^+ and reverse KCC2-dependent transport of this molecule leads to a subsequent intracellular acidification (Titz *et al.* 2006). Rates of steady-state NH_4^+ influx, representing KCC2 activity, were determined by monitoring a 100 s period of the decrease in intracellular pH (Shin *et al.* 2004; Hershfinkel *et al.* 2009).

For imaging of synaptic Zn^{2+} release, slices were first loaded with the Zn^{2+}-selective dye ZinPyr-1 (30 μM, Santa Cruz Biotechnology) for 30 min. Images were then recorded every 10 min with a 20X objective (Olympus, BX51) with 1 × 1 binning (SensiCam, PCO). Higher resolution images were acquired with a FluoView FV1000 confocal system (Olympus).

Statistical significance was determined using t test or ANOVA with *post hoc* comparisons where appropriate.

Surface expression and Immunoblotting

Changes in KCC2 membrane expression were monitored using hippocampal slices as previously described (Rivera *et al.* 2002; Thomas-Crusells *et al.* 2003). Acute slices were biotinylated (100μM sulfo-NHS-Biotin, Pierce) in ACSF (45 min, at room temperature) and then unbound sulfo-NHS-biotin was scavenged using 1 μM lysine in ACSF (X2). For surface expression analysis, biotinylated slices were incubated with kainate (300 nM, 10 min) in the presence or absence of the cell-impermeable Zn^{2+}-chelator CaEDTA (100 μM). Slices maintained in Zn^{2+}-free ACSF for the same time interval were used as controls, detecting the basal time-dependent removal of biotinylated KCC2 from the surface membrane (Rivera *et al.* 2004; Lee *et al.* 2007; Zhao *et al.* 2008). Slices were washed in ACSF and 6 min following kainate, kainate and CaEDTA, CaEDTA alone or control treatment, slices were lysed (1% Triton X-100, 0.1% SDS, 1 mM EDTA, 50 mM NaCl, 20 mM Tris-HCl, pH 7.5, and protease inhibitors, Sigma) and Neutravidin Gel (Pierce) was added (overnight at 4°C), as previously described (Rivera *et al.* 2004). Samples were resolved on 7.5% SDS-PAGE followed by immunoblot analysis of KCC2 (C2366, Sigma) and transferrin receptor (13–6800, Invitrogen), a nonrelated surface protein which was used for normalization. Immunoblots were digitally imaged using ChemImager5 (Alpha-Innotech, Labtrade) and quantified using EZQuant-Gel software.

Results

mZnR/GPR39 KO mice are highly susceptible to kainate induced seizures in-vivo

To evaluate whether mZnR/GPR39 plays a role in regulating seizure activity, adult mouse behavior was monitored for 1 hour following a single i.p. injection of kainic acid (10 mg/kg). In the mature hippocampus, there is a clear link between kainate induced behavioral seizures and cellular effects on gene expression and toxicity in CA3 neurons (Friedman *et al.* 1997; Friedman *et al.* 2008), the cells which putatively express functional mZnR/GPR39 receptors (Besser *et al.* 2009). Seizure severity was graded using a standard severity score in which 0 was “no response”, and 6 was “status epilepticus/death” (see Methods). The maximal seizure severity score during the first hour following kainate injection was significantly higher in mZnR/GPR39 KO mice (Fig. 1A), reaching at least grade 5 (loss of posture or status epilepticus) in 82% of the mice compared to 27% of the WT mice; and grade 6 (status epilepticus) in 27% of the mZnR/GPR39 KO mice versus 9% in the WT mice (Supplemental Video Files 1 and 2). Moreover, seizure activity that was grade 3 (tonic/clonic seizures) or higher lasted longer in mZnR/GPR39 KO mice (average of 22±5.2 min out of 60 min that were monitored; n=8) than in the WT mice (average of 6±5.4 min; n=8; $p<0.05$). We also determined the averaged score across all animals at each time point following application of kainate. As shown in Fig. 1B, seizure score was significantly greater in the mZnR/GPR39 KO mice. To demonstrate the cumulative effect of kainate on seizure activity, strength and duration, we calculated the area under the curve (AUC, integral of trace of seizure severity score vs. time) for each mouse. A significantly higher averaged AUC was demonstrated in mZnR/GPR39 KO mice compared to WT (Fig. 1C). These results

suggest that mZnR/GPR39 may be critical for regulating the severity of seizure activity following kainate treatment *in vivo*.

Persistent gamma oscillations in mZnR/GPR39 KO mice

To test the effect of mZnR/GPR39 on kainate-induced neuronal activity in hippocampal slices, where we have previously characterized the function of the receptor (Chorin *et al.* 2011), we compared field potentials in the CA3 subfield of slices obtained from 15–20 days old WT and mZnR/GPR39 KO mice in the absence and presence of the excitotoxin. Continuous application of a low concentration of kainate (300 nM) resulted in gradual development of oscillatory activity in the gamma (20–70 Hz) range (Fig. 2A), which, as previously reported (Buhl *et al.* 1998), was attenuated by 4 μM bicuculline (Supplemental Fig. 1). Notably, the normalized power spectra in the 25–45 Hz range showed greater activity in kainate-treated slices obtained from mZnR/GPR39 KO mice when compared to WT (Fig. 2A, lower left and center panels). Moreover, chelation of extracellular Zn^{2+} using the non-permeable chelator CaEDTA (100 μM, Fig. 2A, right panel) produced an enhancement of gamma oscillations in WT slices that were similar to those present in slices derived from mZnR/GPR39 KO animals. Indeed, analysis of total AUC of the power spectra at the 25–40 Hz range (normalized to the total area at the full 1–100 Hz range) showed that kainate-induced gamma activity was significantly higher in mZnR/GPR39 KO slices and in CaEDTA treated WT slices, when compared to the activity in control WT slices (Fig. 2B). Finally, Fast Fourier Transformation (FFT, last 300 s of recording) showed that peak frequencies in the 15–50 Hz range were significantly higher in slices from mZnR/GPR39 KO mice compared to WT mice, and that in WT slices treated with CaEDTA the peak frequency was similar to that of the mZnR/GPR39 KO mice (Fig. 2C). These results suggest that the absence of extracellular Zn^{2+} or the lack of mZnR/GPR39 can exacerbate kainate-induced gamma oscillations in the hippocampus. Importantly, in agreement with the *in vivo* actions of kainate on mZnR/GPR39 KO adult mice, the seizure-associated gamma oscillations induced by kainate in slices from P15-P20 mice are enhanced in mZnR/GPR39 KO preparations.

Kainate induces extracellular Zn^{2+}-dependent upregulation of KCC2 activity

To identify a cellular mechanism linking mZnR/GPR39 activity to abnormal electrical activity, we studied neuronal KCC2 function following kainate exposure in slices obtained from mZnR/GPR39 expressing or deficient mice. Fluorescent based imaging of K^+/Cl^- transport was used as a direct measurement of KCC2 activity (Titz *et al.* 2006; Hershfinkel *et al.* 2009; Chorin *et al.* 2011).

First, however, we evaluated whether release of synaptic Zn^{2+} from mossy fiber terminals is triggered by application of 300 nM kainate. Hippocampal slices from WT and mZnR/GPR39 KO P15-P20 mice, pre-loaded with the cell-permeable vesicular Zn^{2+} fluorophore ZinPyr-1 (30 μM), were imaged following the addition of 300 nM kainate. Control slices were perfused with ACSF for the same time period. As shown in Fig. 3A, kainate induced a significant loss of the fluorescence signal, suggestive of synaptic Zn^{2+}-dependent fluorescence loss. Kainate-dependent decrease in fluorescence was quantified using wide field images, allowing for larger fields of view and minimal photobleacing. A nearly two-

fold larger fluorescence decrease ($p<0.05$, Fig. 3B) was measured in slices that were perfused with kainate-containing ACSF compared to controls in WT or mZnR/GPR39 KO slices, suggesting that Zn^{2+} is released to the extracellular region following exposure to the excitotoxin, in agreement with previous studies in older animals using a radioactive Zn^{2+} tracer (Takeda *et al.* 2003).

We then asked if extracellular Zn^{2+}, released during exposure to kainate, enhances KCC2 activity, a mechanism that may be responsible for regulating overall epileptic activity (Woo *et al.* 2002). We treated acute hippocampal slices from WT (C57Bl/6 background) mice with kainate (300 nM, 10 min) and monitored KCC2 activity in BCECF loaded CA3 neurons, as represented by the rate of NH_4^+ transport and acidification (Titz *et al.* 2006; Chorin *et al.* 2011). As shown in Fig. 4A–B, NH_4^+ transport rate was approximately 2.5-fold faster when slices were treated with kainate compared to control. To specifically determine the role of Zn^{2+} in regulation of KCC2 activity we then applied a chelator, CaEDTA (100 μM, Fig. 4A–B), during the kainate exposure. In the presence of the chelator NH_4^+ transport rate was comparable to control conditions, indicating that released synaptic Zn^{2+} is essential to enhance KCC2 activity.

The susceptibility to kainate and subsequent cell death can vary in mice from different genetic backgrounds (McLin *et al.* 2006; Schauwecker 2011). Thus, to determine if Zn^{2+}-dependent regulation of KCC2 activity is a general phenomenon that is not specifically related to the knockdown of mZnR/GPR39 or to the strain used (Moechars *et al.* 2006) we extended our analysis of KCC2 activity to slices from ICR mice, which have been reported to be vulnerable to kainate (McLin *et al.* 2006). In hippocampal slices obtained from these mice we observed an approximately two-fold increase in NH_4^+ transport following exposure to kainate (300 nM, 10 min), when compared to non-treated controls (Fig. 5A, C). The kainate-induced upregulation of NH_4^+ transport was again abolished in the presence of CaEDTA (100 μM) in the perfusate (Fig. 5A–C). Application of a different non-permeable chelator, tricine (10 mM), also prevented the increased NH_4^+ transport induced by kainate (Supplementary Fig. 2). Finally, kainate did not enhance NH_4^+ transport in the presence of the KCC2 inhibitor, [(dihydronindenyl)oxy] alkanoic acid, DIOA (100μM) (Fig. 5B, C), indicating that kainate upregulates KCC2 activity. Thus the effect of kainate and endogenous Zn^{2+} on upregulation of KCC2 activity is similar in slices from WT (C57Bl/6 background) and ICR mice, suggesting the effect is general and likely independent of genetic background. In addition, application of the voltage-gated Na^+ channel blocker tetrodotoxin (TTX, 1 μM) abolished the kainate-dependent enhancement of KCC2 activity (Supplementary Fig. 2), suggesting that it is not mediated directly by the toxin but requires enhanced neuronal activity, as shown previously (Vincent *et al.* 2009; Khirug *et al.* 2010). As such, our results suggest that kainate-dependent enhancement of NH_4^+ transport is dependent on extracellular Zn^{2+}, likely released from presynaptic terminals, as established in the experiments noted earlier.

KCC2 upregulation by kainate depends on IP3 pathway activation

We next asked whether kainate-induced upregulation of KCC2 activity occurred via activation of a signaling pathway that is associated with mZnR/GPR39 function

(Hershfinkel *et al.* 2001; Besser *et al.* 2009; Chorin *et al.* 2011). We measured the rate of NH_4^+ transport in slices from ICR mice treated with kainate (300 nM, 10 min) in the absence or presence of a Gq inhibitor (YM254890, 1 μM, 30 min), a MEK1/2 inhibitor (U0126, 1 μM, 30 min), or a phospholipase-C (PLC) inhibitor (U73122, 1 μM, 30 min). KCC2 activity was also monitored in control slices exposed to kainate in the absence or presence of the vehicle (dimethylsulfoxide, DMSO, 1 μM, 30 min). Consistent with the results shown in Fig. 4, a two-fold increase in NH_4^+ transport rate was monitored in the kainate and vehicle treated slices compared to control slices treated with the vehicle only (Fig. 6A–B). In the presence the Gq inhibitor, the MEK1/2 inhibitor or the PLC inhibitor, the kainate-dependent NH_4^+ transport rates were similar to rates monitored in control slices (Fig. 6A–B). These results strongly suggest that kainate-induced upregulation of KCC2 activity is mediated by mZnR/GPR39-dependent intracellular signaling pathways.

Kainate-dependent KCC2 upregulation is mediated by mZnR/GPR39

To determine directly the role of mZnR/GPR39 in regulating KCC2 activity during kainate exposure, we employed hippocampal slices from mZnR/GPR39 KO mice that were littermates to the WT mice used in Fig. 4. Slices were treated with kainate (300 nM, 10 min) in the presence or absence of the extracellular Zn^{2+} chelator CaEDTA (100 μM, 10 min). In contrast to the effect observed in WT slices expressing mZnR/GPR39, kainate treatment failed to upregulate KCC2-dependent NH_4^+ transport activity in slices from mZnR/GPR39 KO mice (Fig. 7A–B). Moreover, chelation of extracellular Zn^{2+} with CaEDTA did not significantly affect KCC2 activity in slices from mZnR/GPR39 KO mice with or without kainate (Fig. 7A–B). These data suggest that mZnR/GPR39 activation is essential for upregulation of KCC2 activity following kainate exposure.

KCC2 surface expression is rapidly regulated by various stimuli (Rivera *et al.* 2004; Lee *et al.* 2007), including synaptically released Zn^{2+} (Chorin *et al.* 2011; Saadi *et al.* 2012). As such, we investigated whether the kainate-dependent increase in KCC2 activity could be accounted for by an overall increase in KCC2 surface expression. We obtained brain slices containing the hippocampus from WT and mZnR/GPR39 KO mice, and labelled cell surface proteins with biotin, as previously described (Chorin *et al.* 2011). Slices were then incubated for 10 minutes with either ACSF (control), kainate (300 nM), kainate together with CaEDTA (100 μM), or CaEDTA alone. Surface expression of KCC2 was then determined using immunoblots to measure biotinylated KCC2 (BT-KCC2), and normalized to biotinylated transferrin receptor that served as a reference for a surface-expressed protein, as previously described (Khirug *et al.* 2010; Chorin *et al.* 2011). We observed 20±8% increased KCC2 surface expression in WT slices treated with kainate (Fig. 8A, C). This difference was abolished when kainate was applied in the presence of CaEDTA, suggesting extracellular Zn^{2+} is essential for kainate-dependent regulation of KCC2. Finally, KCC2 surface expression was similar in slices treated with CaEDTA alone when compared to control slices, indicating that Zn^{2+} does not affect the baseline expression of this cotransporter. Importantly, in slices from mZnR/GPR39 KO mice kainate did not significantly increase KCC2 surface expression, and CaEDTA showed no effect regardless of the presence or absence of kainate (Fig. 8B, C). These results strongly suggest that upregulation of KCC2 activity following kainate exposure can be accounted, at least in part,

by an overall increase in surface expression of the transporter that is dependent on Zn^{2+} and mZnR/GPR39.

Discussion

Although it is well-recognized that epileptic activity is generally a self-limiting process, the precise molecular, cellular or intercellular events that lead to seizure termination are not completely defined (rev. by (Lado *et al.* 2008)). Here, we uncover a novel mechanism that may be a critical component of the adaptive response to abnormal excitatory activity. We suggest that synaptic Zn^{2+}, released from glutamatergic terminals, activates mZnR/GPR39 and provides an adaptive homeostatic mechanism mediated by enhancement of KCC2 activity (Fig. 9). As the inhibitory $GABA_A$ receptor-mediated drive is critically dependent on sufficient KCC2 activity (Rivera *et al.* 1999; Woo *et al.* 2002; Zhu *et al.* 2005; Viitanen *et al.* 2010; Khalilov *et al.* 2011), an adaptive process that upregulates KCC2 activity may restore the homeostatic balance between excitation and inhibition. In fact, as previous observations suggest that GABA signaling is depolarizing at the onset of seizure activity (Li *et al.* 2008; Miles *et al.* 2012), the mechanism described here may be critically important for restoring inhibitory drive. Indeed, we show that mice lacking mZnR/GPR39, and thereby the homeostatic adaptive KCC2 activity, have substantially more pronounced seizure activity following a single injection of kainic acid. The role of mZnR/GPR39 in a multiple exposure model (e.g. see Claycomb et al. 2012) was not evaluated here, but since the receptor undergoes desensitization by prolonged exposure to Zn^{2+} (Besser *et al.* 2009), frequent exposure to kainate may downregulate its activity. Of interest, it has been recently suggested that indirect or direct strategies that enhance KCC2 function may increase our therapeutic armamentarium in the treatment of epileptic and other neurological disorders (Gagnon *et al.* 2013; Puskarjov *et al.* 2014). Thus, we suggest mZnR/GPR39 as a novel target for therapeutic regulation of the KCC2-dependent adaptive mechanism.

The role of synaptically released Zn^{2+} in regulating seizure activity became first apparent during the description of a phenotype of the vesicular transporter ZnT3 KO mouse (Cole *et al.* 1999). These animals lack synaptic Zn^{2+} and were noted to have substantially enhanced susceptibility to kainate-induced seizures (Cole *et al.* 2000), similar to the phenotype described here for mZnR/GPR39 KO mice. Of note, ZnT3 KO animals presented only a slightly increased threshold to seizure activity triggered by the $GABA_A$ receptor antagonist bicuculine. It was thus suggested that the primary synaptic effect of vesicular Zn^{2+} was to promote inhibition, which is now also supported by our work. In light of the results presented here, the previously described differences in susceptibly to kainate and bicuculine in ZnT3 KO animals can be explained, at least in part, by the fact that Zn^{2+}-dependent enhanced KCC2 activity would be able to attenuate epileptic activity only when $GABA_A$ receptors are active, and would not be as effective when triggered by a drug that blocks $GABA_A$ receptors altogether (bicuculine). An important point is that application of exogenous Zn^{2+} can activate mZnR/GPR39-dependent processes in hippocampal slices obtained from ZnT3 KO mice, indicating that loss of the synaptic Zn^{2+} alone does not abolish mZnR/GPR39 signaling (Besser *et al.* 2009; Chorin *et al.* 2011). In addition, hippocampal slices obtained from mZnR/GPR39 KO mice lack the upregulation of KCC2 transport activity observed in tissue obtained from WT littermates. Taken together, these

results suggest that Zn^{2+} and its receptor mZnR/GPR39 are both essential components of the neuronal adaptation and homeostatic response to seizure activity.

Accumulation of synaptic Zn^{2+} and increased KCC2 expression are both developmentally regulated (Frederickson *et al.* 1981; Lu *et al.* 1999; Penkowa *et al.* 1999; Rivera *et al.* 1999; Nitzan *et al.* 2002; Payne *et al.* 2003; Stein *et al.* 2004). Zn^{2+} release has been difficult to detect in slices from neonatal mice (Frederickson *et al.* 2006) and the absence of KCC2 in these stages yields depolarizing GABA responses (Rivera *et al.* 1999; Lee *et al.* 2005). During the first two weeks of postnatal development, however, synaptic Zn^{2+} accumulation and KCC2 expression reach levels similar to those found in adult mice (Slomianka *et al.* 1997; Nitzan *et al.* 2002; Ludwig *et al.* 2003; Stein *et al.* 2004). Furthermore, at P15–P20 synaptic Zn^{2+} from mossy fibers activates mZnR/GPR39 and upregulates KCC2 activity (Chorin *et al.* 2011), and we show here kainate-dependent Zn^{2+} release at this age. If levels of synaptic Zn^{2+} or KCC2 are increased (Zhu *et al.* 2008) in adult mice the effect of mZnR/GPR39 on KCC2 activity may be augmented, nevertheless it is in agreement with the pronounced effect of mZnR/GPR39 expression on kainate induced seizure *in vivo*.

A single kainate application to neonate slices can induce a hyperpolarizing shift of the reversal potential of $GABA_A$-mediated currents (Khirug *et al.* 2010), and the BDNF/TrkB pathway was recently shown to enhance KCC2 surface expression in this same model (Puskarjov *et al.* 2014). In contrast, in the mature hippocampus, TrkB activation by BDNF during seizure downregulates KCC2 (Rivera *et al.* 2002; Rivera *et al.* 2004). Pharmacological studies note marked differences between neonatal and adult seizures (Loscher *et al.* 2013), suggesting that underlying mechanisms behind seizure generation may be developmentally different. Our results, however, clearly demonstrate that synaptically released Zn^{2+} activates mZnR/GPR39 leading to enhanced KCC2 activity in juvenile slices, and that in the absence of mZnR/GPR39, seizure activity is highly exacerbated in adult mice *in vivo*. Hence this pathway may play a role at the age when TrkB receptors are downregulated and BDNF reduces KCC2 activity (Knusel *et al.* 1994; Di Lieto *et al.* 2012). Although Zn^{2+} has been suggested to influence BDNF signaling (Hwang *et al.* 2005; Huang *et al.* 2008; Travaglia *et al.* 2013; Solati *et al.* 2014), whether the neurotrophin can itself affect mZnR/GPR39 receptor-mediated processes has yet to be determined.

Kainate-induced electrical oscillations have been observed in a variety of preparations, including slices derived from somatosensory cortex and hippocampus (Buhl *et al.* 1998; Traub *et al.* 2004). Gamma oscillatory activity has been linked to ictal activity (Worrell *et al.* 2004) and it arises from interactions between inhibitory and excitatory components of a neural circuit (Kohling *et al.* 2000; Worrell *et al.* 2004; Proddutur *et al.* 2013). We observed that gamma oscillations induced by low concentrations of kainate in WT hippocampal slices were dramatically enhanced by the presence of a Zn^{2+} chelator, similar to the level of kainate-induced oscillatory activity present in slices obtained from mZnR/GPR39 KO mice. These results suggest that synaptic Zn^{2+}, released in the presence of the excitotoxin, and subsequent activation of mZnR/GPR39 serve to dampen this kainate-induced change in hippocampal electrical activity.

Our results demonstrate a 50% increase in KCC2 activity, as measured directly using the BCECF-sensitive NH_4^+ transport assay, compared to only 20% increase in the surface expressed protein, as measured by biotinylation. This difference may be the result of the experimental paradigms utilized, as KCC2 activity measurements are performed in the neuron-enriched CA3 subfield of the hippocampus. In contrast, the surface expression experiment was carried out on biotinylated labeled protein from whole brain slices, probably underestimating the effects within CA3. Finally, although we cannot exclude other molecular pathways that may induce kainate-enhanced KCC2 surface expression, the strong dependence KCC2- activity and surface expression on extracellular Zn^{2+}, as seen by the reversal of these effects by application of CaEDTA, suggests that extracellular Zn^{2+} release is necessary for KCC2 upregulation to occur. Moreover, the lack of the kainate-dependent upregulation on KCC2 in slices from the mZnR/GPR39 KO indicates that mZnR/GPR39 is a major mediator of kainate-dependent KCC2 upregulation in the CA3 region of the hippocampus.

Altogether, our study describes a molecular link between kainate induced excitatory activity and KCC2 upregulation. We show an important role for synaptic Zn^{2+} signaling acting via mZnR/GPR39 to activate an intrinsic homeostatic mechanism that enhances inhibitory drive, likely providing a powerful seizure dampening regulatory process. We suggest that this mechanism could be targeted as a potential novel approach to generating anti-epileptic therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Amaral DG, Scharfman HE, Lavenex P. The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). Prog Brain Res. 2007; 163:3–22. [PubMed: 17765709]

Amaral DG, Witter MP. The three-dimensional organization of the hippocampal formation: a review of anatomical data. Neuroscience. 1989; 31(3):571–591. [PubMed: 2687721]

Baraka AM, Hassab El Nabi W, El Ghotni S. Investigating the role of zinc in a rat model of epilepsy. CNS Neurosci Ther. 2012; 18(4):327–333. [PubMed: 22070383]

Beierlein M, Fall CP, Rinzel J, Yuste R. Thalamocortical bursts trigger recurrent activity in neocortical networks: layer 4 as a frequency-dependent gate. J Neurosci. 2002; 22(22):9885–9894. [PubMed: 12427845]

Besser L, Chorin E, Sekler I, Silverman WF, Atkin S, Russell JT, Hershfinkel M. Synaptically released zinc triggers metabotropic signaling via a zinc-sensing receptor in the hippocampus. J Neurosci. 2009; 29(9):2890–2901. [PubMed: 19261885]

Bischofberger J, Engel D, Li L, Geiger JR, Jonas P. Patch-clamp recording from mossy fiber terminals in hippocampal slices. Nat Protoc. 2006; 1(4):2075–2081. [PubMed: 17487197]

Blaesse P, Airaksinen MS, Rivera C, Kaila K. Cation-chloride cotransporters and neuronal function. Neuron. 2009; 61(6):820–838. [PubMed: 19323993]

Blasco-Ibanez JM, Poza-Aznar J, Crespo C, Marques-Mari AI, Gracia-Llanes FJ, Martinez-Guijarro FJ. Chelation of synaptic zinc induces overexcitation in the hilar mossy cells of the rat hippocampus. Neurosci Lett. 2004; 355(1–2):101–104. [PubMed: 14729245]

Buhl EH, Tamas G, Fisahn A. Cholinergic activation and tonic excitation induce persistent gamma oscillations in mouse somatosensory cortex in vitro. J Physiol. 1998; 513 (Pt 1):117–126. [PubMed: 9782163]

Chorin E, Vinograd O, Fleidervish I, Gilad D, Herrmann S, Sekler I, Aizenman E, Hershfinkel M. Upregulation of KCC2 activity by zinc-mediated neurotransmission via the mZnR/GPR39 receptor. J Neurosci. 2011; 31(36):12916–12926. [PubMed: 21900570]

Cole TB, Robbins CA, Wenzel HJ, Schwartzkroin PA, Palmiter RD. Seizures and neuronal damage in mice lacking vesicular zinc. Epilepsy Res. 2000; 39(2):153–169. [PubMed: 10759303]

Cole TB, Wenzel HJ, Kafer KE, Schwartzkroin PA, Palmiter RD. Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. Proc Natl Acad Sci U S A. 1999; 96(4):1716–1721. [PubMed: 9990090]

Deeb TZ, Lee HH, Walker JA, Davies PA, Moss SJ. Hyperpolarizing GABAergic transmission depends on KCC2 function and membrane potential. Channels (Austin). 2011; 5(6):475–481. [PubMed: 22082832]

Di Lieto A, Rantamaki T, Vesa L, Yanpallewar S, Antila H, Lindholm J, Rios M, Tessarollo L, Castren E. The responsiveness of TrkB to BDNF and antidepressant drugs is differentially regulated during mouse development. PLoS One. 2012; 7(3):e32869. [PubMed: 22396798]

Elsas SM, Hazany S, Gregory WL, Mody I. Hippocampal zinc infusion delays the development of after discharges and seizures in a kindling model of epilepsy. Epilepsia. 2009; 50(4):870–879. [PubMed: 19175668]

Farahani HN, Ashthiani AR, Masihi MS. Study on serum zinc and selenium levels in epileptic patients. Neurosciences (Riyadh). 2013; 18(2):138–142. [PubMed: 23545611]

Frederickson CJ, Giblin LJ 3rd, Balaji RV, Masalha R, Frederickson CJ, Zeng Y, Lopez EV, Koh JY, Chorin U, Besser L, Hershfinkel M, Li Y, Thompson RB, Krezel A. Synaptic release of zinc from brain slices: factors governing release, imaging, and accurate calculation of concentration. J Neurosci Methods. 2006; 154(1–2):19–29. [PubMed: 16460810]

Frederickson CJ, Howell GA, Frederickson MH. Zinc dithizonate staining in the cat hippocampus: relationship to the mossy-fiber neuropil and postnatal development. Exp Neurol. 1981; 73(3):812–823. [PubMed: 6167461]

Frederickson CJ, Koh JY, Bush AI. The neurobiology of zinc in health and disease. Nat Rev Neurosci. 2005; 6(6):449–462. [PubMed: 15891778]

Friedman LK, Saghyan A, Peinado A, Keesey R. Age- and region-dependent patterns of Ca2+ accumulations following status epilepticus. Int J Dev Neurosci. 2008; 26(7):779–790. [PubMed: 18687397]

Friedman LK, Sperber EF, Moshe SL, Bennett MV, Zukin RS. Developmental regulation of glutamate and GABA(A) receptor gene expression in rat hippocampus following kainate-induced status epilepticus. Dev Neurosci. 1997; 19(6):529–542. [PubMed: 9445090]

Gagnon M, Bergeron MJ, Lavertu G, Castonguay A, Tripathy S, Bonin RP, Perez-Sanchez J, Boudreau D, Wang B, Dumas L, Valade I, Bachand K, Jacob-Wagner M, Tardif C, Kianicka I, Isenring P, Attardo G, Coull JA, De Koninck Y. Chloride extrusion enhancers as novel therapeutics for neurological diseases. Nat Med. 2013; 19(11):1524–1528. [PubMed: 24097188]

Ganesh R, Janakiraman L. Serum zinc levels in children with simple febrile seizure. Clin Pediatr (Phila). 2008; 47(2):164–166. [PubMed: 17873242]

Goldberg HJ, Sheehy EM. Fifth day fits: an acute zinc deficiency syndrome? Arch Dis Child. 1982; 57(8):633–635. [PubMed: 7114883]

Hershfinkel M, Kandler K, Knoch ME, Dagan-Rabin M, Aras MA, Abramovitch-Dahan C, Sekler I, Aizenman E. Intracellular zinc inhibits KCC2 transporter activity. Nat Neurosci. 2009; 12(6):725–727. [PubMed: 19430470]

Hershfinkel M, Moran A, Grossman N, Sekler I. A zinc-sensing receptor triggers the release of intracellular Ca2+ and regulates ion transport. Proc Natl Acad Sci U S A. 2001; 98(20):11749–11754. [PubMed: 11573009]

Huang YZ, Pan E, Xiong ZQ, McNamara JO. Zinc-Mediated Transactivation of TrkB Potentiates the Hippocampal Mossy Fiber-CA3 Pyramid Synapse. Neuron. 2008; 57(4):546–558. [PubMed: 18304484]

Huberfeld G, Wittner L, Clemenceau S, Baulac M, Kaila K, Miles R, Rivera C. Perturbed chloride homeostasis and GABAergic signaling in human temporal lobe epilepsy. J Neurosci. 2007; 27(37): 9866–9873. [PubMed: 17855601]

Hwang JJ, Park MH, Choi SY, Koh JY. Activation of the Trk Signaling Pathway by Extracellular Zinc: Role of Metalloproteinases. J Biol Chem. 2005; 280(12):11995–12001. [PubMed: 15659400]

Jackson VR, Nothacker HP, Civelli O. GPR39 receptor expression in the mouse brain. Neuroreport. 2006; 17(8):813–816. [PubMed: 16708020]

Kahle KT, Deeb TZ, Puskarjov M, Silayeva L, Liang B, Kaila K, Moss SJ. Modulation of neuronal activity by phosphorylation of the K-Cl cotransporter KCC2. Trends Neurosci. 2013; 36(12):726–737. [PubMed: 24139641]

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. Front Cell Neurosci. 2011; 5:23. [PubMed: 22065950]

Khirug S, Ahmad F, Puskarjov M, Afzalov R, Kaila K, Blaesse P. A single seizure episode leads to rapid functional activation of KCC2 in the neonatal rat hippocampus. J Neurosci. 2010; 30(36): 12028–12035. [PubMed: 20826666]

Knusel B, Rabin SJ, Hefti F, Kaplan DR. Regulated neurotrophin receptor responsiveness during neuronal migration and early differentiation. J Neurosci. 1994; 14(3 Pt 2):1542–1554. [PubMed: 8126554]

Kohling R, Vreugdenhil M, Bracci E, Jefferys JG. Ictal epileptiform activity is facilitated by hippocampal GABAA receptor-mediated oscillations. J Neurosci. 2000; 20(18):6820–6829. [PubMed: 10995826]

Lado FA, Moshe SL. How do seizures stop? Epilepsia. 2008; 49(10):1651–1664. [PubMed: 18503563]

Lee H, Chen CX, Liu YJ, Aizenman E, Kandler K. KCC2 expression in immature rat cortical neurons is sufficient to switch the polarity of GABA responses. Eur J Neurosci. 2005; 21(9):2593–2599. [PubMed: 15932617]

Lee HH, Walker JA, Williams JR, Goodier RJ, Payne JA, Moss SJ. Direct protein kinase C-dependent phosphorylation regulates the cell surface stability and activity of the potassium chloride cotransporter KCC2. J Biol Chem. 2007; 282(41):29777–29784. [PubMed: 17693402]

Li X, Jefferys JG, Fox J, Yao X. Neuronal population oscillations of rat hippocampus during epileptic seizures. Neural Netw. 2008; 21(8):1105–1111. [PubMed: 18657392]

Li Y, Hough CJ, Frederickson CJ, Sarvey JM. Induction of mossy fiber --> Ca3 long-term potentiation requires translocation of synaptically released Zn2+ J Neurosci. 2001; 21(20):8015–8025. [PubMed: 11588174]

Loscher W, Puskarjov M, Kaila K. Cation-chloride cotransporters NKCC1 and KCC2 as potential targets for novel antiepileptic and antiepileptogenic treatments. Neuropharmacology. 2013; 69:62–74. [PubMed: 22705273]

Lu J, Karadsheh M, Delpire E. Developmental regulation of the neuronal-specific isoform of K-Cl cotransporter KCC2 in postnatal rat brains. J Neurobiol. 1999; 39(4):558–568. [PubMed: 10380077]

Ludwig A, Li H, Saarma M, Kaila K, Rivera C. Developmental up-regulation of KCC2 in the absence of GABAergic and glutamatergic transmission. Eur J Neurosci. 2003; 18(12):3199–3206. [PubMed: 14686894]

McLin JP, Steward O. Comparison of seizure phenotype and neurodegeneration induced by systemic kainic acid in inbred, outbred, and hybrid mouse strains. Eur J Neurosci. 2006; 24(8):2191–2202. [PubMed: 17074044]

Miles, R.; Blaesse, P.; Huberfeld, G.; Wittner, L.; Kaila, K. Chloride homeostasis and GABA signaling in temporal lobe epilepsy. 2012.

Moechars D, Depoortere I, Moreaux B, de Smet B, Goris I, Hoskens L, Daneels G, Kass S, Ver Donck L, Peeters T, Coulie B. Altered gastrointestinal and metabolic function in the GPR39-obestatin receptor-knockout mouse. Gastroenterology. 2006; 131(4):1131–1141. [PubMed: 17030183]

Nitzan YB, Sekler I, Hershfinkel M, Moran A, Silverman WF. Postnatal regulation of ZnT-1 expression in the mouse brain. Brain Res Dev Brain Res. 2002; 137(2):149–157. [PubMed: 12220707]

Paoletti P, Vergnano AM, Barbour B, Casado M. Zinc at glutamatergic synapses. Neuroscience. 2008; 15:15.

Payne JA, Rivera C, Voipio J, Kaila K. Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci. 2003; 26(4):199–206. [PubMed: 12689771]

Penkowa M, Nielsen H, Hidalgo J, Bernth N, Moos T. Distribution of metallothionein I + II and vesicular zinc in the developing central nervous system: correlative study in the rat. J Comp Neurol. 1999; 412(2):303–318. [PubMed: 10441758]

Perez-Rosello T, Anderson CT, Schopfer FJ, Zhao Y, Gilad D, Salvatore SR, Freeman BA, Hershfinkel M, Aizenman E, Tzounopoulos T. Synaptic Zn2+ inhibits neurotransmitter release by promoting endocannabinoid synthesis. J Neurosci. 2013; 33(22):9259–9272. [PubMed: 23719795]

Peterlin ZA, Kozloski J, Mao B-Q, Tsiola A, Yuste R. Optical probing of neuronal circuits with calcium indicators. Proceedings of the National Academy of Sciences. 2000; 97(7):3619–3624.

Proddutur A, Yu J, Elgammal FS, Santhakumar V. Seizure-induced alterations in fast-spiking basket cell GABA currents modulate frequency and coherence of gamma oscillation in network simulations. Chaos. 2013; 23(4):046109. [PubMed: 24387588]

Puskarjov M, Ahmad F, Khirug S, Sivakumaran S, Kaila K, Blaesse P. BDNF is required for seizure-induced but not developmental up-regulation of KCC2 in the neonatal hippocampus. Neuropharmacology. 2014

Puskarjov M, Kahle KT, Ruusuvuori E, Kaila K. Pharmacotherapeutic targeting of cation-chloride cotransporters in neonatal seizures. Epilepsia. 2014; 55(6):806–818. [PubMed: 24802699]

Puskarjov M, Seja P, Heron SE, Williams TC, Ahmad F, Iona X, Oliver KL, Grinton BE, Vutskits L, Scheffer IE, Petrou S, Blaesse P, Dibbens LM, Berkovic SF, Kaila K. A variant of KCC2 from patients with febrile seizures impairs neuronal Cl− extrusion and dendritic spine formation. EMBO Rep. 2014; 15(6):723–729. [PubMed: 24668262]

Qian J, Noebels JL. Visualization of transmitter release with zinc fluorescence detection at the mouse hippocampal mossy fibre synapse. J Physiol. 2005; 566(Pt 3):747–758. [PubMed: 15919713]

Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol. 1972; 32(3):281–294. [PubMed: 4110397]

Rivera C, Li H, Thomas-Crusells J, Lahtinen H, Viitanen T, Nanobashvili A, Kokaia Z, Airaksinen MS, Voipio J, Kaila K, Saarma M. BDNF-induced TrkB activation down-regulates the K+-Cl− cotransporter KCC2 and impairs neuronal Cl− extrusion. J Cell Biol. 2002; 159(5):747–752. [PubMed: 12473684]

Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K. The K+/Cl− co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature. 1999; 397(6716):251–255. [PubMed: 9930699]

Rivera C, Voipio J, Thomas-Crusells J, Li H, Emri Z, 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. 2004; 24(19):4683–4691. [PubMed: 15140939]

Saad K, Hammad E, Hassan AF, Badry R. Trace element, oxidant, and antioxidant enzyme values in blood of children with refractory epilepsy. Int J Neurosci. 2014; 124(3):181–186. [PubMed: 23919524]

Saadi RA, He K, Hartnett KA, Kandler K, Hershfinkel M, Aizenman E. SNARE-dependent upregulation of potassium chloride co-transporter 2 activity after metabotropic zinc receptor activation in rat cortical neurons in vitro. Neuroscience. 2012; 210:38–46. [PubMed: 22441041]

Schauwecker PE. The relevance of individual genetic background and its role in animal models of epilepsy. Epilepsy Res. 2011; 97(1–2):1–11. [PubMed: 22001434]

Sensi SL, Paoletti P, Bush AI, Sekler I. Zinc in the physiology and pathology of the CNS. Nat Rev Neurosci. 2009; 10(11):780–791. [PubMed: 19826435]

Sensi SL, Paoletti P, Koh JY, Aizenman E, Bush AI, Hershfinkel M. The neurophysiology and pathology of brain zinc. J Neurosci. 2011; 31(45):16076–16085. [PubMed: 22072659]

Seven M, Basaran SY, Cengiz M, Unal S, Yuksel A. Deficiency of selenium and zinc as a causative factor for idiopathic intractable epilepsy. Epilepsy Res. 2013; 104(1–2):35–39. [PubMed: 23103062]

Shin JH, Namkung W, Choi JY, Yoon JH, Lee MG. Purinergic stimulation induces Ca2+-dependent activation of Na+-K+-2Cl− cotransporter in human nasal epithelia. J Biol Chem. 2004; 279(18): 18567–18574. [PubMed: 14982922]

Slomianka L, Geneser FA. Postnatal development of zinc-containing cells and neuropil in the hippocampal region of the mouse. Hippocampus. 1997; 7(3):321–340. [PubMed: 9228529]

Smart TG, Hosie AM, Miller PS. Zn2+ ions: modulators of excitatory and inhibitory synaptic activity. Neuroscientist. 2004; 10(5):432–442. [PubMed: 15359010]

Solati Z, Jazayeri S, Tehrani-Doost M, Mahmoodianfard S, Gohari MR. Zinc monotherapy increases serum brain-derived neurotrophic factor (BDNF) levels and decreases depressive symptoms in overweight or obese subjects: A double-blind, randomized, placebo-controlled trial. Nutr Neurosci. 2014

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. 2004; 468(1):57–64. [PubMed: 14648690]

Takeda A, Hirate M, Tamano H, Oku N. Zinc movement in the brain under kainate-induced seizures. Epilepsy Res. 2003; 54(2–3):123–129. [PubMed: 12837563]

Thomas-Crusells J, Vieira A, Saarma M, Rivera C. A novel method for monitoring surface membrane trafficking on hippocampal acute slice preparation. J Neurosci Methods. 2003; 125(1–2):159–166. [PubMed: 12763242]

Titz S, Hormuzdi S, Lewen A, Monyer H, Misgeld U. Intracellular acidification in neurons induced by ammonium depends on KCC2 function. Eur J Neurosci. 2006; 23(2):454–464. [PubMed: 16420452]

Traub RD, Bibbig A, LeBeau FE, Buhl EH, Whittington MA. Cellular mechanisms of neuronal population oscillations in the hippocampus in vitro. Annu Rev Neurosci. 2004; 27:247–278. [PubMed: 15217333]

Travaglia A, La Mendola D, Magri A, Pietropaolo A, Nicoletti VG, Grasso G, Malgieri G, Fattorusso R, Isernia C, Rizzarelli E. Zinc(II) interactions with brain-derived neurotrophic factor N-terminal peptide fragments: inorganic features and biological perspectives. Inorg Chem. 2013; 52(19): 11075–11083. [PubMed: 24070197]

Veliskova J, Claudio OI, Galanopoulou AS, Lado FA, Ravizza T, Velisek L, Moshe SL. Seizures in the developing brain. Epilepsia. 2004; 45(Suppl 8):6–12. [PubMed: 15610187]

Vergnano AM, Rebola N, Savtchenko LP, Pinheiro PS, Casado M, Kieffer BL, Rusakov DA, Mulle C, Paoletti P. Zinc dynamics and action at excitatory synapses. Neuron. 2014; 82(5):1101–1114. [PubMed: 24908489]

Viitanen T, Ruusuvuori E, Kaila K, Voipio J. The K+-Cl cotransporter KCC2 promotes GABAergic excitation in the mature rat hippocampus. J Physiol. 2010; 588(Pt 9):1527–1540. [PubMed: 20211979]

Vincent P, Mulle C. Kainate receptors in epilepsy and excitotoxicity. Neuroscience. 2009; 158(1):309–323. [PubMed: 18400404]

Vogt K, Mellor J, Tong G, Nicoll R. The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron. 2000; 26(1):187–196. [PubMed: 10798403]

Wojciak RW, Mojs E, Stanislawska-Kubiak M, Samborski W. The serum zinc, copper, iron, and chromium concentrations in epileptic children. Epilepsy Res. 2013; 104(1–2):40–44. [PubMed: 23103060]

Woo NS, Lu J, England R, McClellan R, Dufour S, Mount DB, Deutch AY, Lovinger DM, Delpire E. Hyperexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. Hippocampus. 2002; 12(2):258–268. [PubMed: 12000122]

Worrell GA, Parish L, Cranstoun SD, Jonas R, Baltuch G, Litt B. High-frequency oscillations and seizure generation in neocortical epilepsy. Brain. 2004; 127(Pt 7):1496–1506. [PubMed: 15155522]

Zhao B, Wong AY, Murshid A, Bowie D, Presley JF, Bedford FK. Identification of a novel di-leucine motif mediating K(+)/Cl(−) cotransporter KCC2 constitutive endocytosis. Cell Signal. 2008; 20(10):1769–1779. [PubMed: 18625303]

Zhu L, Lovinger D, Delpire E. Cortical neurons lacking KCC2 expression show impaired regulation of intracellular chloride. J Neurophysiol. 2005; 93(3):1557–1568. [PubMed: 15469961]

Zhu L, Polley N, Mathews GC, Delpire E. NKCC1 and KCC2 prevent hyperexcitability in the mouse hippocampus. Epilepsy Res. 2008; 79(2–3):201–212. [PubMed: 18394864]

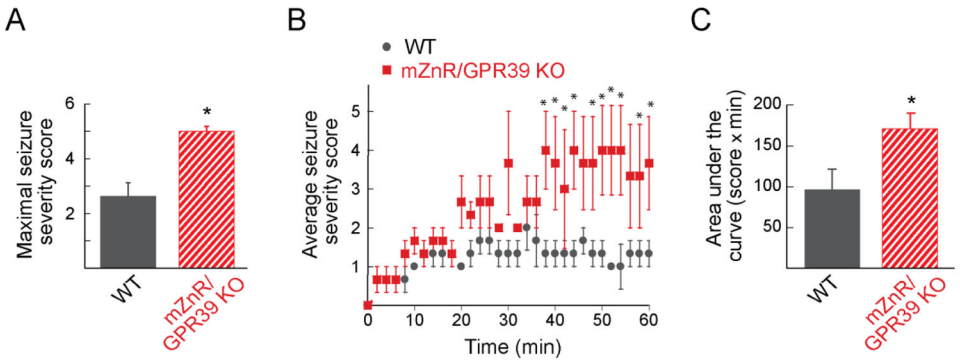

Fig. 1. mZnR/GPR39 KO mice are highly susceptible to kainate-induced seizures

A. The maximal seizure severity score reached by a mouse at any time during a 1 hr observation period following a single intraperitoneal injection of kainic acid (10 mg/kg) was averaged across all mice for each phenotype. This represents the maximal effect of kainate in triggering seizures (n=8 for both groups, P=0.03). B. Averaged seizure severity score as a function of time across all mice tested of each genotype. Kainate injection is at time 0, and significantly higher seizure scores are monitored for the mZnR/GPR39 KO mice from 40 min. following the injection. We noted increased response variability in mZnR/GPR39 KO mice vs. WT. C. Area under the curve (AUC, score x min) was calculated for individual plots of seizure score vs. time for each mouse using the integral of the curve. Averaged AUC over all mice from each genotype represent the cumulative effect of kainate (P=0.01).

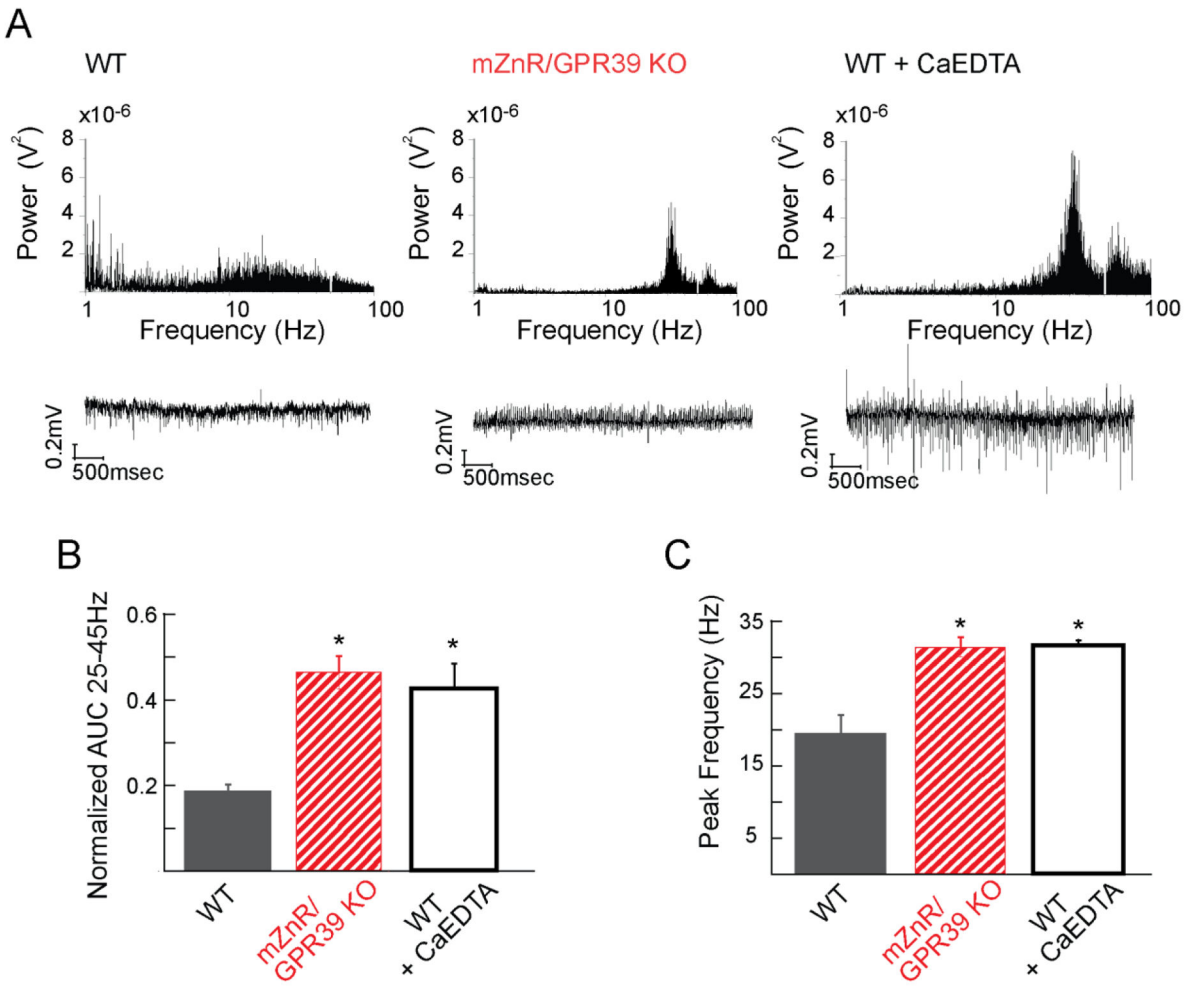

Fig. 2. Absence of mZnR/GPR39 or extracellular Zn^{2+} exacerbates kainate-induced gamma frequency oscillations in hippocampal slices

A. Extracellular field potentials were monitored in the stratum pyramidale of CA3 in slices from WT and mZnR/GPR39 KO mice in the continuous presence of kainate (300 nM), or in slices from WT animals treated with kainate in the presence of the extracellular Zn^{2+} chelator CaEDTA (100 μM). The upper panels show representative power spectra for the signal recorded between 35 and 40 minutes following initial exposure to kainate in single slices. Note that a resonant 50 Hz peak (electrical power supply) was excluded in all analyses. Lower panels show representative 5s of raw data that generated the corresponding power spectra in the upper panels. B. Averaged data showing the area under the power spectrum curve (AUC) between 25–45Hz normalized to AUC at 1–100Hz. C. The frequency of maximal power between 10–45Hz as obtained from FFT analysis (for both B and C n=7; * $p<0.0001$; ANOVA, vs. WT group).

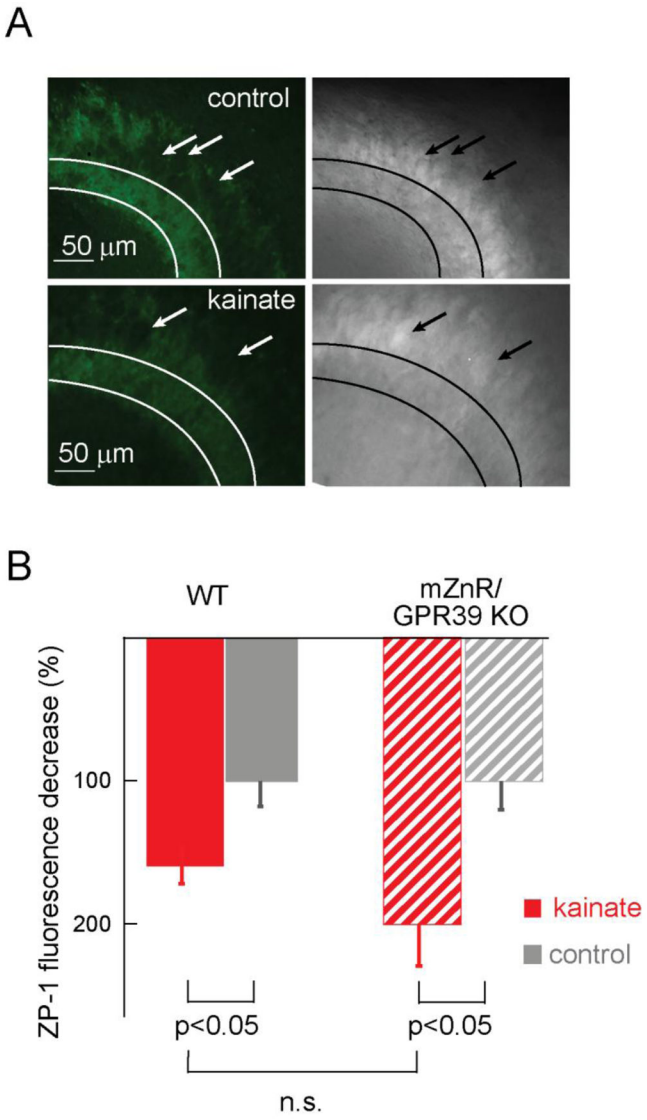

Fig. 3. Kainate induces a decrease in Zn^{2+}-dependent fluorescence from mossy fiber terminals in both WT and mZnR/GPR39 KO mice

A. Hippocampal slices loaded with the specific Zn^{2+} fluorescent indicator ZnPyr-1 were treated with either ACSF vehicle (control) or 300 nM kainate. Representative fluorescence images (left panels) and the corresponding bright-field images (right panels) are shown. Note that mossy fiber terminals subfield exhibits high intensity fluorescence signals (lines), most likely representing vesicular Zn^{2+}; while neuronal somas lack staining (arrows). B. Differences between overall fluorescent signal in the terminal region subfield before and after application of kainate in slices from WT and mZnR/GPR39 KO mice. Note that 100% represents the average loss of staining in slices treated with vehicle only, which likely results from bleaching and loss of dye during the course of the experiment (n=6 for each group, *p<0.05 ANOVA, vs. WT control).

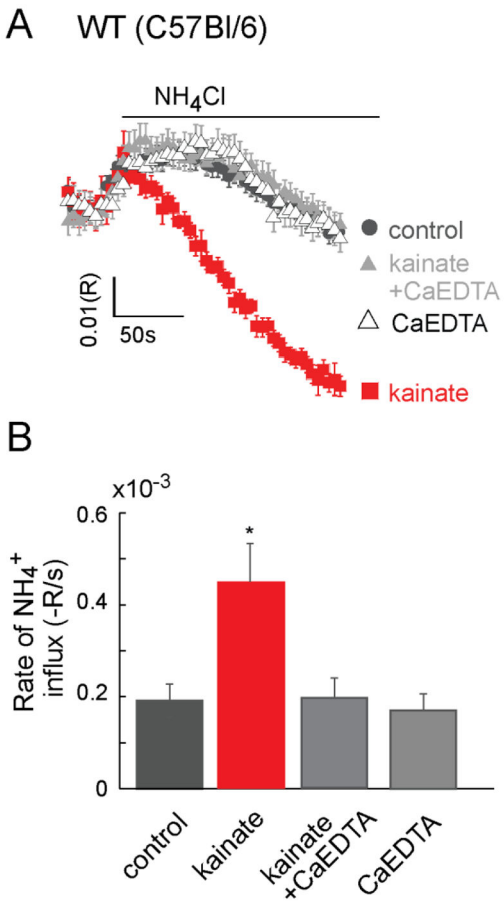

Fig. 4. Upregulation of KCC2 activity by kainate is dependent on extracellular Zn^{2+}

A. Hippocampal slices (WT C57BL/6 background) were treated with kainate (300 nM) and/or an extracellular Zn^{2+} chelator, CaEDTA (100 μM), and KCC2 activity was monitored using the NH_4Cl/BCECF paradigm (see Materials and Methods). Averaged traces of BCECF fluorescence over population of ROIs from a single representative slice are shown. B. Averaged rates of initial NH_4^+ influx (acidification) under the various conditions shown in A (n=7–8 slices per each experimental paradigm; * $p<0.01$, ANOVA, vs. Control).

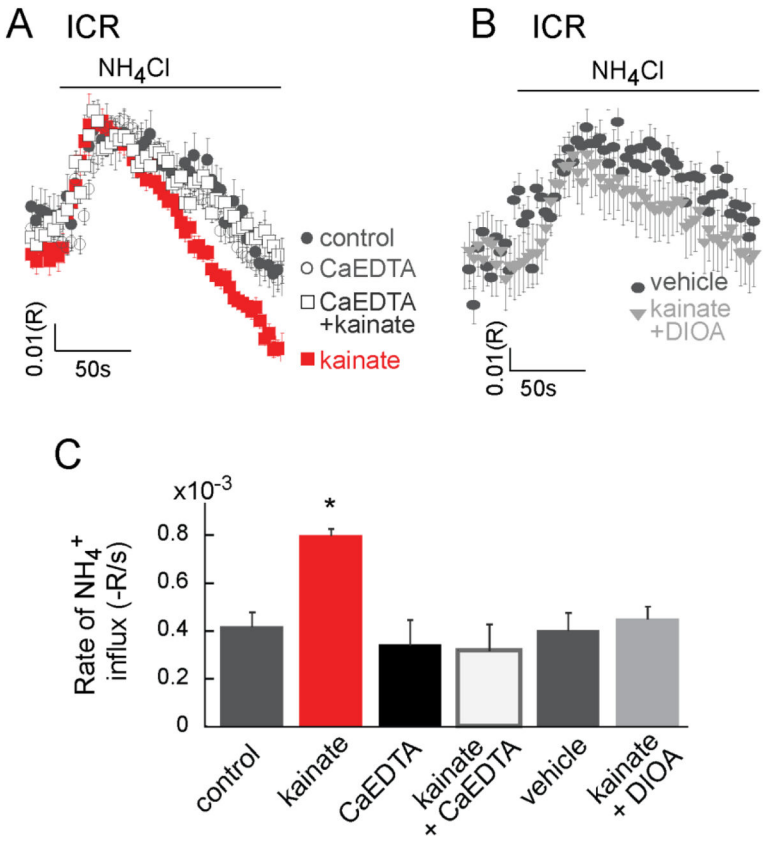

Fig. 5. Upregulation of KCC2 activity by kainate is dependent on extracellular Zn^{2+}

A. Traces of BCECF fluorescence from representative hippocampal slices (averaged over population of ROIs from a single slice) from ICR mice treated with kainate (300 nM) in the presence or absence of CaEDTA (100 μM) are shown (as in Fig. 4A). B. KCC2 activity in vehicle (1% DMSO) or kainate-treated slices in the presence of the KCC2 inhibitor DIOA (100 μM). C. Averaged rates of initial NH_4^+ influx (acidification) under the various conditions shown in A–B (n=5–6 slices per each experimental paradigm; * $p<0.01$, ANOVA, vs. Control).

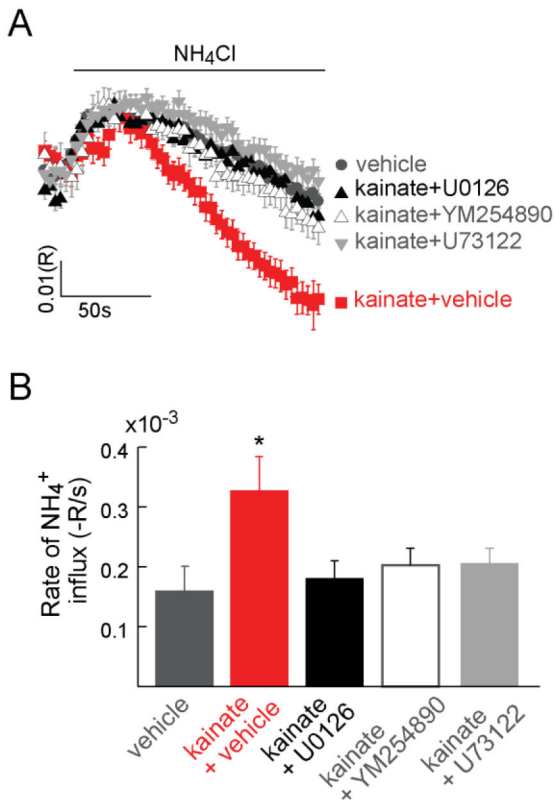

Fig. 6. Upregulation of KCC2 activity following application of kainate is dependent on Gq/PLC/ERK signaling

Hippocampal slices (ICR background) were treated with kainate (300 nM) alone or in the presence of either a Gq inhibitor (YM254890, 1 μM), a PLC inhibitor (U73122, 1 μM) or a MEK1/2 (ERK pathway) inhibitor (U0126, 1 μM) for 30 minutes prior to imaging using the BCECF paradigm. Slices treated with vehicle (1% DMSO) alone were used as controls. A. Representative traces of changes in BCECF fluorescence, as described in Fig. 4, following each of the treatments are shown. B. Averaged rates of NH_4^+ influx for the different groups (n=5 for each treatment, * $p<0.01$; ANOVA, vs. vehicle).

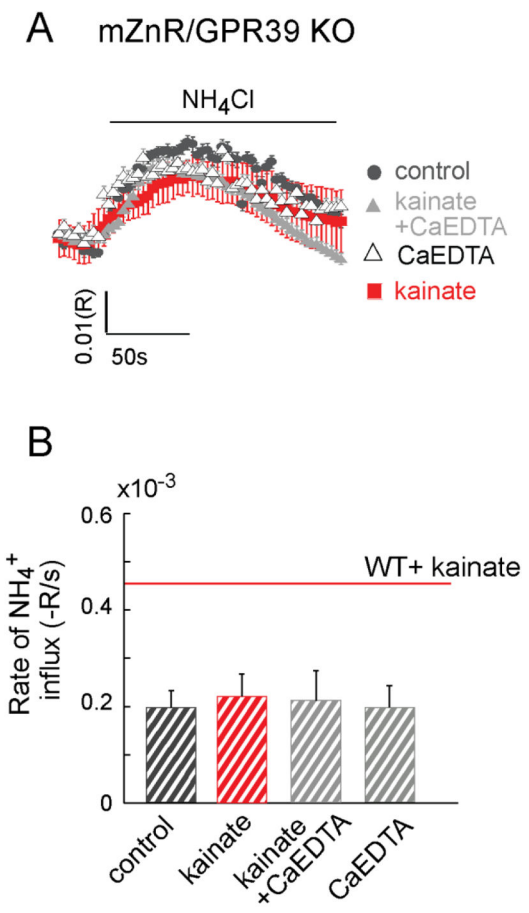

Fig. 7. Upregulation of KCC2 activity by kainate is dependent on mZnR/GPR39

A. Hippocampal slices from mZnR/GPR39 KO mice were loaded with BCECF and treated with kainate (300 nM, 10 min) in the absence or presence of the extracellular Zn^{2+} chelator, CaEDTA (100 μM, 10 min) prior to imaging using the BCECF paradigm. Representative traces of changes in BCECF fluorescence, as described in Fig. 4, for each group are shown. B. Averaged rates of NH_4^+ transport, reflecting KCC2 activity (n=8–9 slices per each experimental paradigm, *p<0.05, vs. WT control). Line marks the rate of NH_4^+ influx in WT mice treated with kainate (taken from Fig. 4).

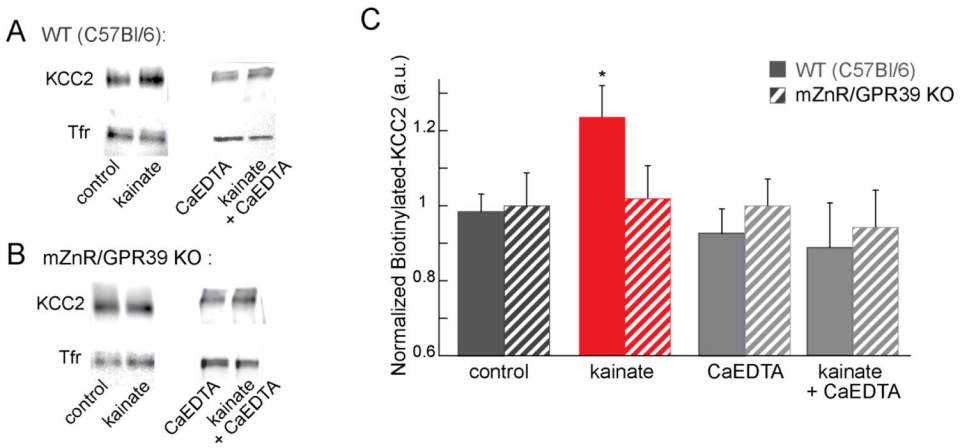

Fig. 8. Extracellular Zn^{2+} and mZnR/GPR39 are required to induce increases in KCC2 surface expression following kainate treatment

A. Surface expression levels of KCC2 were monitored using surface biotinylation followed by immunoblotting of KCC2 or transferrin receptor (Tfr), used as surface protein marker control. Brain slices containing the hippocampus from WT (top panel) or mZnR/GPR39 KO (bottom panel) mice were treated with or without kainate (300 nM, 10 min) in the presence or absence of CaEDTA (100 μM). Non-consecutive lanes from the same blot are delimited by a gap. B. Densitometry analysis of KCC2 surface expression normalized to the expression in control slices. Tfr surface expression was used as loading control (n=11; *p<0.05 vs. WT control).

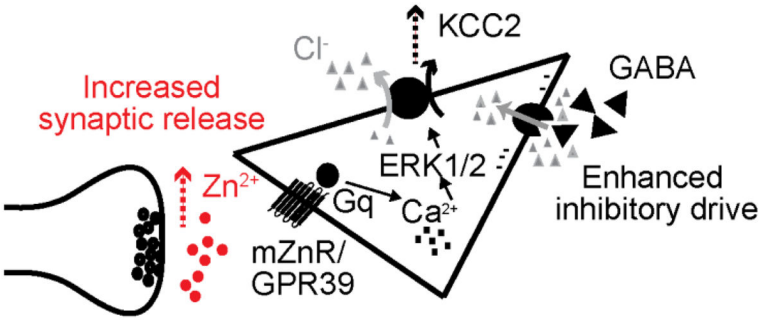

Fig. 9. Schematic illustration describing the interpretation of our findings

Activation of mZnR/GPR39 by sustained/increased excitatory activity, resulting from a single KA exposure (300 nM, 10 min), triggers a Gq-mediated signaling pathways leading to ERK activation and enhanced KCC2 surface expression. A likely consequence of this process is decreased $[Cl^-]_i$ and enhanced inhibitory drive resulting in an adaptive homeostatic mechanism for regulation of the excitatory/inhibitory balance. Note that further studies to determine the effective exposure to kainate that will maximize KCC2 surface expression and activity may provide an even stronger effect.