



Homeostatic regulation of KCC2 activity by the zinc receptor mZnR/GPR39 during seizures

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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 $G\alpha_q$ /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.

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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-specific zinc transporter ZnT3 (Cole et al., 1999, 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 and Noebels, 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, 2000), and importantly, deficiencies in plasma Zn^{2+} levels have been associated with human epileptic disorders (Goldberg and Sheehy, 1982; Blasco-Ibanez et al., 2004; Ganesh and Janakiraman, 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^- co-transporter 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

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hyperpolarizing GABA_A receptor-mediated synaptic potentials (Rivera et al., 2002, 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., 2014a,b,c). 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 induces a KCC2-dependent shift in GABA_A reversal potential in neonatal rat hippocampus (Khurug et al., 2010). Here, we show a regulatory feedback process of KCC2 activity that is triggered via Zn²⁺-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 Zn²⁺ 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'-ACCTCAT CT TGGTGACCT-3' and 5'-TGAGCGCTCAAAGCTGAG-3' amplified a 311-bp band from the WT allele, whereas primers 5'-GGAACCTCACT CGACCTGGG-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 h. Statistical analysis was performed using Mann Whitney U-test.

Slice preparation

Hippocampal slices were obtained from postnatal days 15–20 (P15–P20) mice of either sex, following protocols aimed to preserve the mossy fiber structure (Amaral and Witter, 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 (400 μm) were kept in artificial CSF (ACSF in mM: 124 NaCl,

26 NaHCO₃, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 3 KCl, and 10 glucose, pH = 7.4 ± 0.1) at room temperature. To test the effects of extracellular Zn²⁺, slices were transferred to NaH₂PO₄-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 Zn²⁺-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-10 L 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). The 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 the 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 Zn²⁺ are found in the hippocampus at levels similar to those of adult neurons (Slomianka and Geneser, 1997; Rivera et al., 1999; Nitzan et al., 2002; Stein et al., 2004), thus allowing the measurements of the effects of kainate-dependent Zn²⁺ 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 sub-field of the hippocampus, were acquired every 3 s (Imaging Workbench 4, INDEC BioSysPhotonics) using a 10× 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 NH₄Cl (10 mM) to the slice, which induced rapid initial alkalinization due to passive entry of NH₃ into the cells (Titz et al., 2006). Extracellular NH₄⁺ 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 NH₄⁺ 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 Zn²⁺ release, slices were first loaded with the Zn²⁺-selective dye ZinPyr-1 (30 μM, Santa Cruz Biotechnology) for 30 min. Images were then recorded every 10 min with a 20× 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 (100 μ M sulfo-NHS-Biotin, Pierce) in ACSF (45 min, at room temperature) and then unbound sulfo-NHS-biotin was scavenged using 1 μ M 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 μ M). The 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). The 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 non-related 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 h 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, 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 Materials and methods section). 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, the 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 the 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 the 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 the field potentials in the CA3 sub-field 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 μ M 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 μ M, 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, the analysis of total AUCs 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

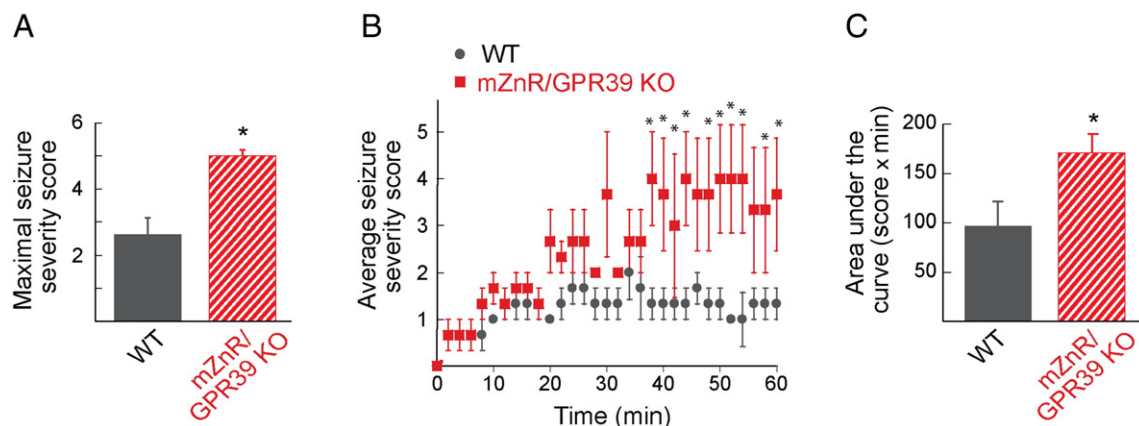


Fig. 1. mZnR/GPR39 KO mice are highly susceptible to kainate-induced seizures. A. The maximal seizure severity score reached by a mouse at any time during a 1 h observation period following a single intraperitoneal injection of kainic acid (10 mg/kg) was averaged across all mice for each phenotype. This 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 \times min) was calculated for individual plots of seizure score vs. time for each mouse using the integral of the curve. Averaged AUCs over all mice from each genotype represent the cumulative effect of kainate ($p = 0.01$).

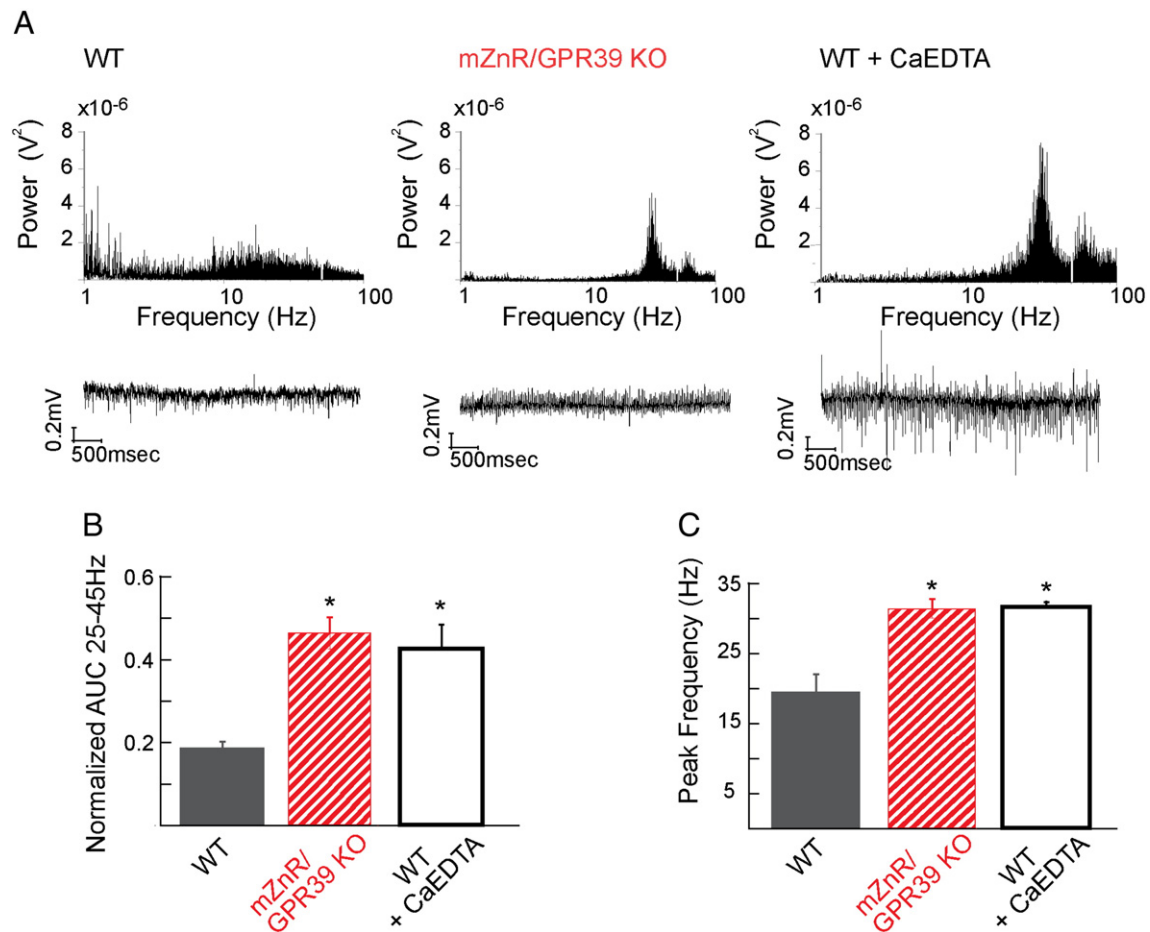


Fig. 2. Absence of mZnR/GPR39 or extracellular Zn^{2+} exacerbates kainate-induced gamma frequency oscillations in hippocampal slices. **A.** Extracellular field potentials were monitored in the stratum pyramidale of CA3 in slices from WT and mZnR/GPR39 KO mice in the continuous presence of kainate (300 nM), or in slices from WT animals treated with kainate in the presence of the extracellular Zn^{2+} chelator CaEDTA (100 μ M). The upper panels show representative power spectra for the signal recorded between 35 and 40 min 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 5 s 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 25–45 Hz normalized to AUC at 1–100 Hz. **C.** The frequency of maximal power between 10–45 Hz as obtained from FFT analysis (for both B and C $n = 7$; * $p < 0.0001$; ANOVA, vs. WT group).

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 the 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 the neuronal KCC2 function following kainate exposure in the 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; Hershinkel et al., 2009; Chorin et al., 2011).

First, however, we evaluated whether the release of synaptic Zn^{2+} from mossy fiber terminals is triggered by the application of 300 nM kainate. The hippocampal slices from WT and mZnR/GPR39 KO P15–P20 mice, pre-loaded with the cell-permeable vesicular Zn^{2+} fluorophore ZinPyr-1 (30 μ M), 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 widefield images, allowing for larger fields of view and minimal photobleaching. 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 the 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 Figs. 4A–B, NH_4^+ transport rate was approximately 2.5-fold faster when the slices were treated with kainate compared to the control. To specifically determine the role of Zn^{2+} in the regulation of KCC2 activity we then applied a chelator, CaEDTA (100 μ M, Figs. 4A–B), during the kainate exposure. In the presence of the chelator the NH_4^+ transport rate was comparable to the control conditions, indicating that the 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 and Steward, 2006;

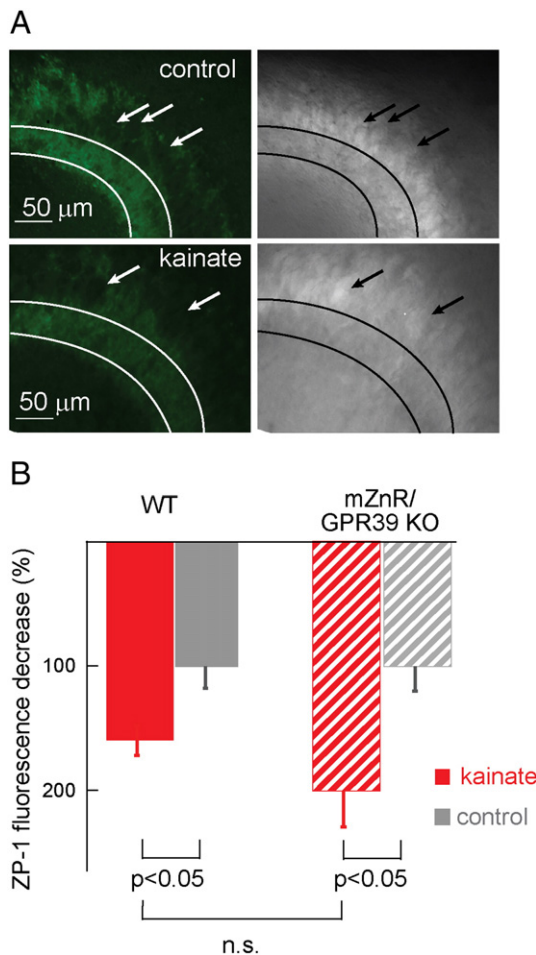


Fig. 3. Kainate induces a decrease in Zn^{2+} -dependent fluorescence from mossy fiber terminals in both WT and mZnR/GPR39 KO mice. **A.** Hippocampal slices loaded with the specific Zn^{2+} fluorescent indicator ZnPy-1 were treated with either ACSF vehicle (control) or 300 nM kainate. Representative fluorescence images (left panels) and the corresponding bright-field images (right panels) are shown. Note that mossy fiber terminals sub-field 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 sub-field 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).

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 the slices from the ICR mice, which have been reported to be vulnerable to kainate (McLin and Steward, 2006). In the hippocampal slices obtained from these mice we observed an approximately two-fold increase in the NH_4^+ transport following exposure to kainate (300 nM, 10 min), when compared to the non-treated controls (Figs. 5A, C). The kainate-induced upregulation of NH_4^+ transport was again abolished in the presence of CaEDTA (100 μM) in the perfusate (Figs. 5A–C). The 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 [(dihydropyridyl)oxy] alkanic acid, DIOA (100 μM) (Figs. 5B, C), indicating that kainate upregulates KCC2 activity. Thus the effect of kainate and endogenous Zn^{2+} on the upregulation of KCC2 activity is similar in the slices from WT (C57Bl/6 background) and ICR mice, suggesting that the effect is general and likely independent of genetic background. In addition, the application of the voltage-gated

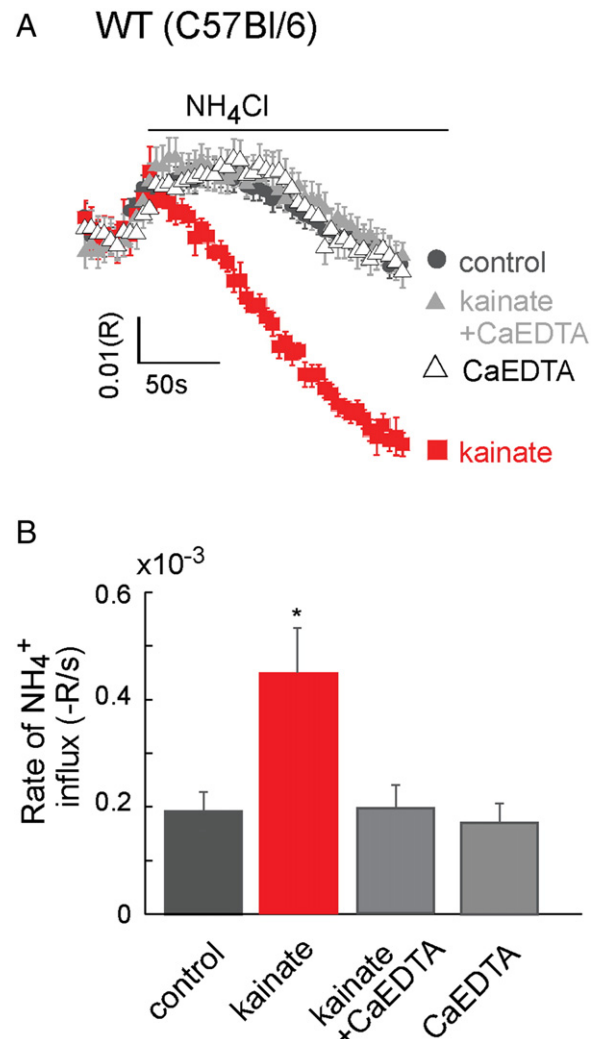


Fig. 4. Upregulation of KCC2 activity by kainate is dependent on extracellular Zn^{2+} . **A.** Hippocampal slices (WT C57Bl/6 background) were treated with kainate (300 nM) and/or an extracellular Zn^{2+} chelator, CaEDTA (100 μM), and KCC2 activity was monitored using the $\text{NH}_4\text{Cl}/\text{BCECF}$ paradigm (see Materials and methods) section. 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).

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 and Mulle, 2009; Khirug et al., 2010). As such, our results suggest that kainate-dependent enhancement of the 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 the activation of a signaling pathway that is associated with mZnR/GPR39 function (Hershinkel et al., 2001; Besser et al., 2009; Chorin et al., 2011). We measured the rate of NH_4^+ transport in the slices from the 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 the control slices exposed to kainate in the absence or presence of the vehicle (dimethylsulfoxide, DMSO, 1 μM , 30 min). Consistent with the

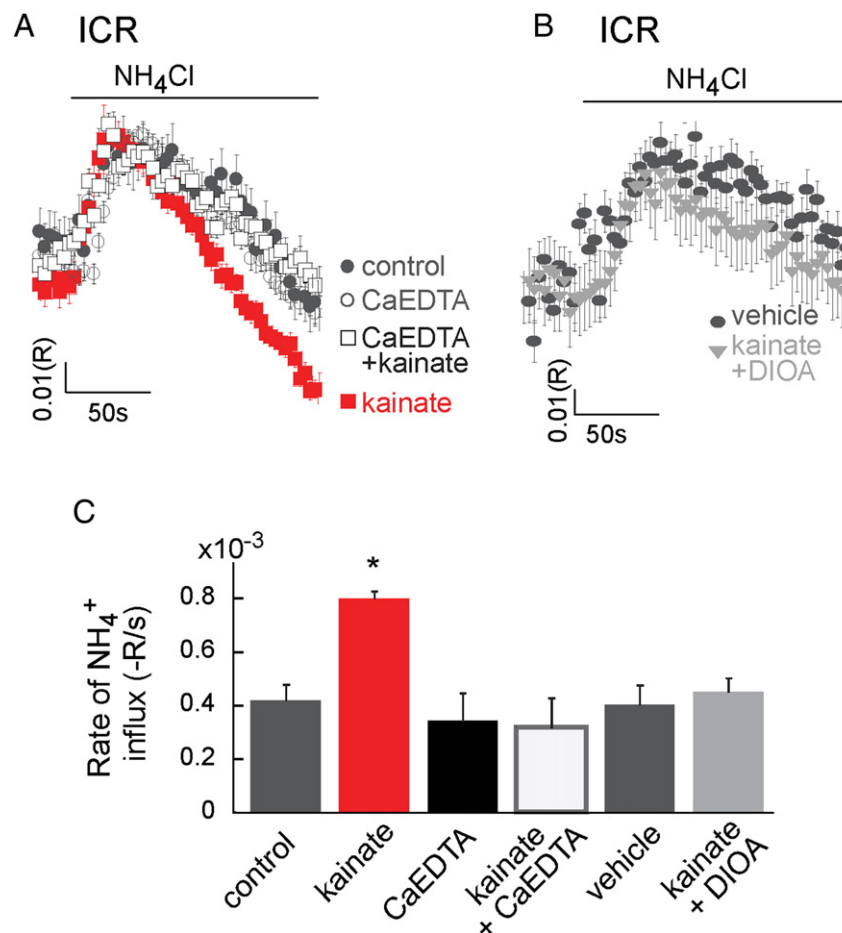


Fig. 5. Upregulation of KCC2 activity by kainate is dependent on extracellular Zn^{2+} . A. Traces of BCECF fluorescence from representative hippocampal slices (averaged over population of ROIs from a single slice) from ICR mice treated with kainate (300 nM) in the presence or absence of CaEDTA (100 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).

results shown in Fig. 4, a two-fold increase in the NH_4^+ transport rate was monitored in the kainate and vehicle treated slices compared to the control slices treated with the vehicle only (Figs. 6A–B). In the presence of the Gq inhibitor, the MEK1/2 inhibitor or the PLC inhibitor, the kainate-dependent NH_4^+ transport rates were similar to the rates monitored in the control slices (Figs. 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 the hippocampal slices from mZnR/GPR39 KO mice that were littermates to the WT mice used in Fig. 4. The 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 the WT slices expressing mZnR/GPR39, the kainate treatment failed to upregulate KCC2-dependent NH_4^+ transport activity in the slices from the mZnR/GPR39 KO mice (Figs. 7A–B). Moreover, the chelation of extracellular Zn^{2+} with CaEDTA did not significantly affect KCC2 activity in the slices from mZnR/GPR39 KO mice with or without kainate (Figs. 7A–B). These data suggest that mZnR/GPR39 activation is essential for the 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 the brain slices containing the hippocampus from WT and mZnR/GPR39 KO mice, and labeled cell surface proteins with biotin, as previously described (Chorin et al., 2011). The slices were then incubated for 10 min with either ACSF (control), kainate (300 nM), kainate together with CaEDTA (100 μM), or CaEDTA alone. The 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 (Khurug et al., 2010; Chorin et al., 2011). We observed $20 \pm 8\%$ increased KCC2 surface expression in the WT slices treated with kainate (Figs. 8A, C). This difference was abolished when kainate was applied in the presence of CaEDTA, suggesting that extracellular Zn^{2+} is essential for the kainate-dependent regulation of KCC2. Finally, the KCC2 surface expression was similar in the slices treated with CaEDTA alone when compared to the control slices, indicating that Zn^{2+} does not affect the baseline expression of this cotransporter. Importantly, in the slices from mZnR/GPR39 KO mice kainate did not significantly increase the KCC2 surface expression, and CaEDTA showed no effect regardless of the presence or absence of kainate (Figs. 8B, C). These results strongly suggest that the upregulation of KCC2 activity following kainate exposure can be accounted, at least in part, by an overall increase in the surface expression of the transporter that is dependent on Zn^{2+} and mZnR/GPR39.

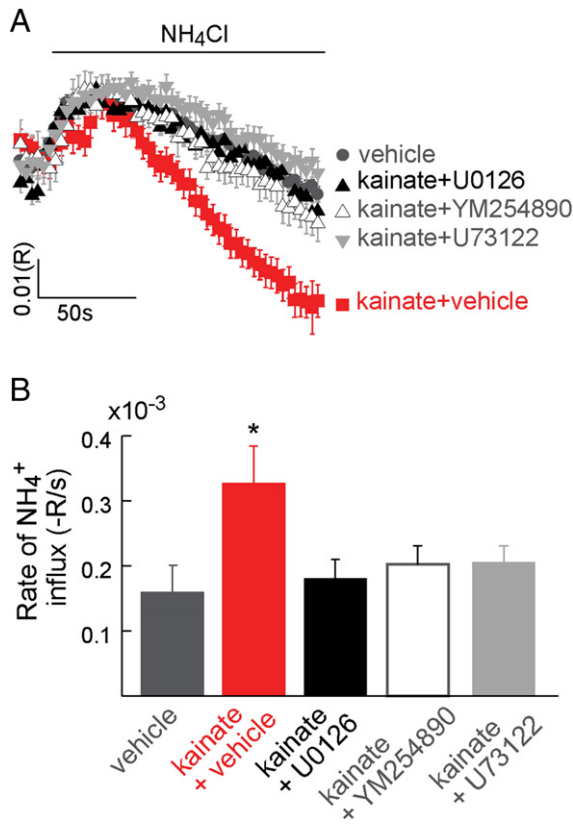


Fig. 6. Upregulation of KCC2 activity following application of kainate is dependent on Gq/PLC/ERK signaling. Hippocampal slices (ICR background) were treated with kainate (300 nM) alone or in the presence of either a Gq inhibitor (YM254890, 1 μ M), a PLC inhibitor (U73122, 1 μ M) or a MEK1/2 (ERK pathway) inhibitor (U0126, 1 μ M) for 30 min 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 NH₄⁺ influx for the different groups (n = 5 for each treatment, * p < 0.01; ANOVA, vs. vehicle).

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 and Moshe, 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 Zn²⁺, released from glutamatergic terminals, activates mZnR/GPR39 and provides an adaptive homeostatic mechanism mediated by the 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 Zn²⁺ (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

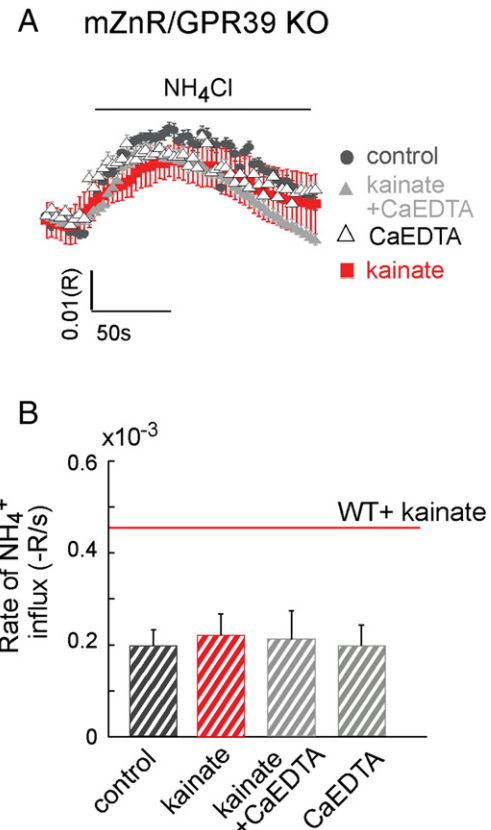


Fig. 7. Upregulation of KCC2 activity by kainate is dependent on mZnR/GPR39. A. Hippocampal slices from mZnR/GPR39 KO mice were loaded with BCECF and treated with kainate (300 nM, 10 min) in the absence or presence of the extracellular Zn²⁺ chelator, CaEDTA (100 μ M, 10 min) prior to imaging using the BCECF paradigm. Representative traces of changes in BCECF fluorescence, as described in Fig. 4, for each group are shown. B. Averaged rates of NH₄⁺ transport, reflecting KCC2 activity (n = 8–9 slices per each experimental paradigm, * p < 0.05, vs. WT control). Line marks the rate of NH₄⁺ influx in WT mice treated with kainate. Taken from Fig. 4.

disorders (Gagnon et al., 2013; Puskarjov et al., 2014a,b,c). Thus, we suggest mZnR/GPR39 as a novel target for the therapeutic regulation of the KCC2-dependent adaptive mechanism.

The role of synaptically released Zn²⁺ 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 Zn²⁺ and were noted to have substantially enhanced susceptibility to kainate-induced seizures (Cole et al., 2000), similar to the phenotype described here for the 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 Zn²⁺ was to promote inhibition, which is now also supported by our work. In light of the results presented here, the previously described differences in susceptibility to kainate and bicuculine in ZnT3 KO animals can be explained, at least in part, by the fact that Zn²⁺-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 the application of exogenous Zn²⁺ can activate the mZnR/GPR39-dependent processes in the hippocampal slices obtained from the ZnT3 KO mice, indicating that the loss of the synaptic Zn²⁺ alone does not abolish the mZnR/GPR39 signaling (Besser et al., 2009; Chorin et al., 2011). In addition, the hippocampal slices obtained from the mZnR/GPR39 KO mice lack the upregulation of KCC2 transport activity observed in the tissue obtained from the WT littermates. Taken together, these results suggest that

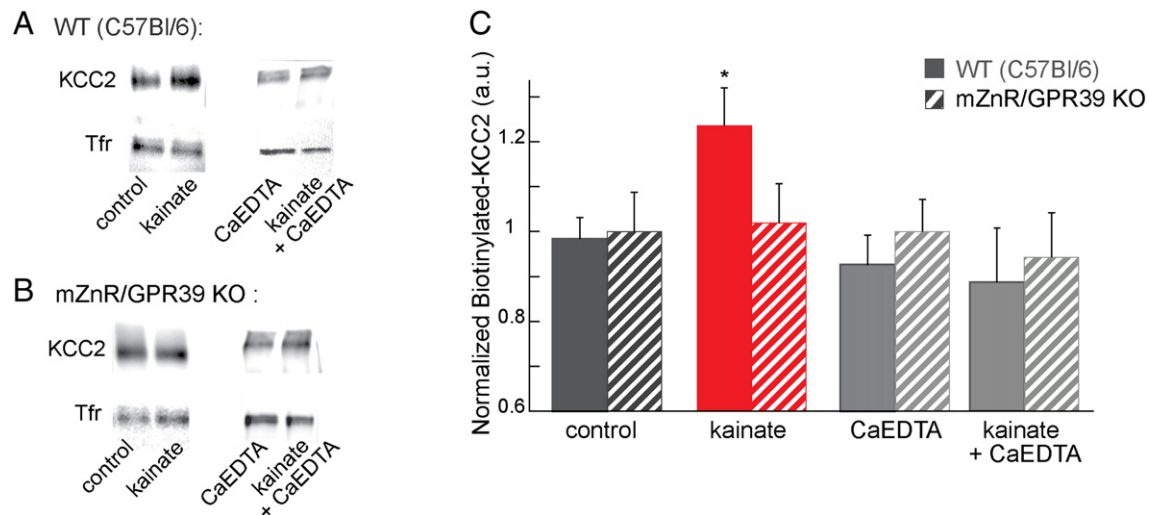


Fig. 8. Extracellular Zn^{2+} and mZnR/GPR39 are required to induce increases in KCC2 surface expression following kainate treatment. A. Surface expression levels of KCC2 were monitored using surface biotinylation followed by immunoblotting of KCC2 or transferrin receptor (Tfr), used as surface protein 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 μ M). 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 a loading control ($n = 11$; * $p < 0.05$ vs. WT control).

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). The Zn^{2+} release has been difficult to detect in the 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 and Geneser, 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 the 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 the KCC2 activity may be augmented, nevertheless it is in agreement with the pronounced effect of the 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

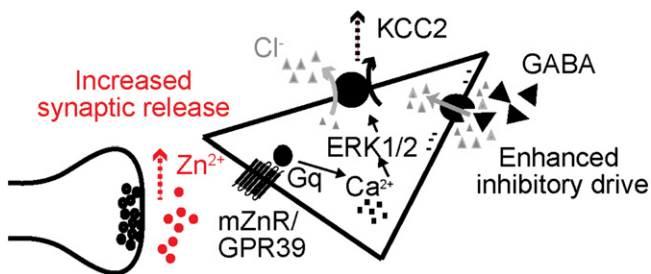


Fig. 9. Schematic illustration describing the interpretation of our findings. Activation of mZnR/GPR39 by sustained/increased excitatory activity, resulting from a single KA exposure (300 nM, 10 min), triggers a Gq-mediated signaling pathways leading to ERK activation and enhanced KCC2 surface expression. A likely consequence of this process is decreased $[Cl^-]$ 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.

(Khirug et al., 2010), and the BDNF/TrkB pathway was recently shown to enhance KCC2 surface expression in this same model (Puskarjov et al., 2014a,b,c). In contrast, in the mature hippocampus, TrkB activation by BDNF during seizure downregulates KCC2 (Rivera et al., 2002, 2004). Pharmacological studies note marked differences between the neonatal and adult seizures (Loscher et al., 2013), suggesting that the 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 the 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 the mZnR/GPR39 receptor-mediated processes has yet to be determined.

Kainate-induced electrical oscillations have been observed in a variety of preparations, including the slices derived from the 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 the 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 the 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 the slices obtained from the 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 the hippocampal electrical activity.

Our results demonstrate a 50% increase in the 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 the KCC2 activity measurements are performed in the neuron-enriched CA3 sub-field of the hippocampus. In contrast, the surface expression experiment was carried out on the biotinylated labeled protein from whole brain slices, probably

underestimating the effects within CA3. Finally, although we cannot exclude other molecular pathways that may induce the kainate-enhanced KCC2 surface expression, the strong dependence of the KCC2-activity and surface expression on extracellular Zn^{2+} , as seen by the reversal of these effects by the application of CaEDTA, suggests that the extracellular Zn^{2+} release is necessary for KCC2 upregulation to occur. Moreover, the lack of the kainate-dependent upregulation on KCC2 in the slices from the mZnR/GPR39 KO indicates that mZnR/GPR39 is a major mediator of the kainate-dependent KCC2 upregulation in the CA3 region of the hippocampus.

Altogether, our study describes a molecular link between the 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 the 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 data to this article can be found online at <http://dx.doi.org/10.1016/j.nbd.2014.12.020>.

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