Supporting Information

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SI Materials and Methods

Animal Care. All animal studies were performed with protocols approved by the Institutional Animal Care and Use Committee of Tufts New England Medical Center.

Behavioral Assessments. The open field task provides a measure of the baseline activity levels of an animal, as well as its habituation response to the novel environment. Activity levels were monitored for 30 min. The rotarod task, measuring the length of time an animal can stay on a moving rod, provides an indication of the motor coordination of that animal. Mice were tested at three different speeds: 18, 24, and 28 rpm. These experiments were performed as described previously (1).

Cultured Neuron Preparation, Immunoprecipitation, Immunoblotting, and Immunohistochemistry. Hippocampal neurons were dissected from rodent embryos at embryonic day 18 as described previously. Cultures were maintained in neurobasal medium supplemented with B-27 neural supplement and 2 mM l-glutamine for 3 wk before the experiments. KCC2 was immunoprecipitated from detergent-solubilizied brain extracts using the respective primary antibodies conjugated to protein A-Sepharose. Immunoblots were visualized using ECL and quantified using a Fuji LAS 3000 imager. Hippocampal sections were stained with KCC2 antibodies followed by HRP-conjugated secondary antibodies and visualized with DAB staining. Images were taken with an Olympus BX51 microscope at 10× magnification and quantified with Metamorph software (Molecular Devices). The methods were as previously described (1, 2). For Western blotting, Neuromab 75-013 anti-KCC2 was used, and pS940 antibody was obtained from PhosphoSolutions (p1551-940) (3). Tubulin and actin antibodies were from Sigma.

Electrophysiology.

Low magnesium extracellular field recordings. Horizontal slices (400 μM) containing the entorhinal cortex and hippocampus were prepared from 3- to 5-wk-old male C57BL6 WT ($n = 8$) and S940A ($n = 3$) mice. Mice were anesthetized with isoflurane and brains were removed and cut in ice-cold cutting solution on a Leica VT1000s vibratome. The cutting solution contained (in millimoles): NaCl 126, NaHCO3 26, NaH_2PO_4 1.25, KCl 2.5, MgCl2 2, CaCl2 0.5, Na-pyruvate 1.5, and glucose 10. Slices were placed in a submerged chamber for a 60-min recovery period at 32 °C in normal ACSF containing (in millimoles): NaCl 126, NaHCO3 26, KCl 5, MgCl2 2, CaCl2 2, glutamine 1, NaH_2PO_4 1.25, Na-pyruvate 1.5, and glucose 10. Slices were then transferred to a Warner Instruments recording chamber (RC-27L) and perfused for 15 min with normal ACSF (2 mL/min, 32 °C) before changing to ACSF containing no $MgCl_2$. All solutions were bubbled with 95% (vol/vol) O_2/5% (vol/vol) CO_2 gas mixture. Field recordings were made with NaCl (154 mM) filled electrodes from the medial entorhinal cortex layers III/IV. Recordings were performed with a Multiclamp 700B amplifier (Molecular Devices) with Clampex 10 acquisition software (Molecular Devices). Data were filtered at 10 kHz and analyzed offline with Clampfit (Molecular Devices).

Cultured neuron recordings. Bath saline contained (in millimoles) 140 NaCl, 2.5 KCl, 2.5 CaCl2, 1.2 MgCl2, 10 Hepes, 11 glucose, pH 7.4 NaOH. Stocks of furosemide were dissolved in DMSO (0.1% final concentration), glutamate in 1 M HCl (in saline, balanced with vol/vol 1 M NaOH), and AP5 in 1 M NaOH (in saline, balanced with vol/vol 1 M HCl); all of these compounds were purchased from Tocris Bioscience. We used a three-barreled (0.7-mm diameter per barrel) glass perfusion pipette placed just above the target neuron to apply all compounds and control saline. These solutions were applied through the perfusion pipette at a rate of 0.5 mL/min and we used a computer-controlled perfusion fast-step device (Warner Instruments) to ensure fast and complete exchange of solutions. To ensure that solutions equilibrated faster, the only source of solution was the perfusion pipette; the bath inlet pipe was not flowing.

Obtaining E_{GABA} values. In acute slices, we performed steady-state current–voltage protocols to obtain E_{GABA} values. Glass micropipettes were filled with 10 μM muscimol, which was applied locally by pressure ejection with a picospritzer to activate $GABA_A$-mediated currents at voltages between −100 to −50 mV in 10-mV increments. In cultured neurons, we used voltage-ramp protocols to determine E_{GABA} values. We first performed a voltage ramp in the absence of muscimol to measure the background leak currents, followed 10 s later by a second voltage ramp in the presence of muscimol. We ramped the holding potential from −100 to −70 mV or −90 to −60 mV (20 mV/s) when muscimol was hyperpolarizing and we ramped the holding potential from −60 to −30 mV (20 mV/s) when muscimol was depolarizing. We performed off-line leak subtraction to isolate the muscimol-activated currents, which we plotted against the holding potential to obtain I–V relationships. For both steady-state and voltage-ramp procedures, we then obtained E_{GABA} values from linear regression fits to the data at voltages near the observable reversal potential. All slice and cultured neuron recordings were performed using the perforated patch-clamp technique. We used gramicidin D (50 μg/mL, Sigma Aldrich, final DMSO concentration was 0.1%) to establish access resistances between 20 and 60 MΩ throughout the recording period. All recordings were performed at 34 °C. The recording pipettes were filled with saline containing (in millimoles) 140 KCl and 10 Hepes, pH 7.4 KOH. Recordings were performed with a 700A Multiclamp amplifier or a 200B Axopatch amplifier (Molecular Devices) and data were acquired with Clampex 10 software and analyzed with Clampfit. All records were filtered at 2 kHz and digitized at 10 kHz.

Selection criteria of cultured neurons. All of the data are presented as replicates of experiments performed on the same cultured hippocampal 18–21 DIV neurons. Due to the variability of neurons in culture, we only patched pyramidal-shaped neurons. We also used adapted selection criteria (4). Briefly, if neurons did not meet all of these criteria they were discarded. Neurons had to maintain membrane potential values less than −50 mV in I = 0 mode to ensure health; neurons had to reestablish membrane potential values less than −50 mV within 1 min after exposure to glutamate or furosemide to ensure proper function of the Na^+/K^+ ATPase; and neurons had to exhibit GABAergic hyperpolarizing postsynaptic potentials and muscimol responses under basal conditions.

Protocols to obtain rates of Cl^- extrusion. The neurons were patched and monitored for basal activity to determine if they met the selection criteria as mentioned in the preceding section. The following sequence was performed for cells exposed to glutamate: (*i*) measure E_{GABA} in voltage-clamp mode, (*ii*) obtain basal muscimol responses for 2 min (500-ms pulses spaced 20 s apart) in I = 0 mode, (*iii*) three 10-s pulses of glutamate in I = 0 mode, (*iv*) measure the muscimol responses every 20 s in control saline for the first 20 s and in the presence of TTX for 4 min in I = 0 mode, (*v*) measure E_{GABA} in voltage-clamp mode, (*vi*) washout TTX for 5 min while recording in I = 0 mode, (*vii*) three 10-s pulses of glutamate in I = 0 mode, and (*viii*) measure E_{GABA} in voltage-clamp mode. The following sequence was performed for

cells exposed to furosemide: (*i*) measure E_{GABA} in voltage-clamp mode, (*ii*) obtain basal muscimol responses for 2 min (500-ms pulses spaced 20 s apart) in I = 0 mode, (*iii*) apply furosemide for 3 min in I = 0 mode, (*iv*) measure E_{GABA} in voltage-clamp mode, (*v*) measure the muscimol responses every 20 s in control saline for the first 20 s and then in the presence of TTX for 4 min in I = 0 mode, and (*vi*) measure E_{GABA} in voltage-clamp mode. We used the following procedure to measure rates of Cl^- extrusion from the peaks of the muscimol responses obtained in I = 0 mode. The peak values of three consecutive muscimol pulses were averaged for the normalized basal value for each cell. The peak of the muscimol pulse after glutamate or furosemide was then used as the normalized loaded value for each cell. The subsequent peaks obtained after application of TTX were then calculated by the following equation: 100% × (post-TTX value − basal value)/(loaded value − basal value). Therefore, as the neurons reestablish hyperpolarizing muscimol responses and normal levels of intracellular Cl^-, the peaks of the muscimol responses (post-TTX value) will approach the basal value and the above function will approach 0, indicating complete recovery. These normalized percentages were then plotted for graphical representation. The rates were calculated by linear regression analysis and expressed as percent recovery per minute. We obtained rates during the course of the linear phase of recovery for each neuron between 40 and 100 s after washout of glutamate or furosemide to avoid the slower phase, which indicates that the system is approaching thermodynamic equilibrium. Using the perforated patch clamp technique, one cannot measure KCC2-mediated Cl^- efflux when the system reaches equilibrium.

Generation and Genotyping of Transgenic Mice. S940A mice were generated at genOway. Briefly, Flp-mediated excision enabled deletion of the neomycin selection cassette, resulting in a *Slc12a5* point-mutation knockin allele. This deletion was performed in vivo by breeding the recombined animals with ubiquitous Flp-recombinase expressing "delete mice." PCR and Southern blot screening were used to distinguish the *Slc12a5* wild-type and NEO-excised knockin alleles. S940A mice were backcrossed on the C57 background before experimentation. WT littermates were used as controls in all experiments.

Induction of SE and EEG Recordings. Age-matched 8-wk WT and S940A mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. An EEG/electromyography (EMG) implant was placed on the skull above lambda and four screws were used as leads. The implant was glued to the skull and the mice were allowed to recover for 7 d. Baseline EEG recordings were started 30 min before an i.p. injection of 20 mg/kg kainate (Sigma) and continued for more than 120 min. Measures of seizure susceptibility were the seizure latency, latency to the onset of SE, the cumulative time seizing expressed as a percentage of the total recording time, and the average duration of individual electrographic events. Seizure latency was defined as the time elapsed from the injection of kainate to the start of the first electrographic seizure. The durations of individual epileptiform events were measured from the start of the epileptiform discharge until return to baseline. Epileptiform activity was defined as discharges with amplitudes greater than five times the SD of the baseline noise. The average durations of these events observed over 120 min were averaged to obtain the average seizure duration and the onset of SE. The SE onset was defined as the time at which the last gap of epileptiform activity that was greater than 2 min occurred, i.e., SE developed when all subsequent epileptiform discharges were spaced less than 2 min apart.

Slice Biotinylation. WT mice were deeply anesthetized and killed by decapitation 30 min after kainate administration and subsequent entrance into SE. To prepare slices from S940A mice, mice were decapitated before death after ∼25 min of SE. At this point, tonic-clonic whole body convulsions would cease, the mouse became immobile, and died within 30 s. The 350-μm slices were prepared using a vibratome (VT1000S, Leica) in ice-cold ACSF containing (in millimoles): 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 2 $MgCl_2$, and 10 glucose with 95% O_2/5% CO_2 to ensure physiological pH of 7.4. Slices were allowed to recover for 1 h at 32 °C to allow for the reequilibration of basal levels of proteins and postmortem-induced changes in phosphorylation. Slices were transferred to ice-cold ACSF solution with 1 mg/mL cleavable NHS-SS-biotin for a 30-min incubation. Excess biotin was quenched with glycine buffer. Slices were washed in ACSF, lysed with 1× RIPA lysis buffer containing protease inhibitors, and incubated overnight with streptavidin beads. Protein was eluted off the beads with SDS and separated by SDS/PAGE followed by Western blot. Samples of total protein (preavidin pull down) were run as controls to determine variations in protein extraction and surface protein pull-down efficiency between slices. In addition, the abundance of tubulin in biotinylated samples was used as an indicator of slice damage; samples with anything less than a 20-fold reduction in the expression levels of these intracellular proteins in the biotin-labeled cell membrane fraction were discarded. Transferrin receptor protein (TfR), an abundant cell surface protein, was used as a loading control.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 4 software. Unpaired *t* tests are presented unless otherwise indicated, and one- or two-way ANOVA were used where indicated. Data were expressed as mean ± SEM.

1. Tretter V, et al. (2009) Deficits in spatial memory correlate with modified gamma-aminobutyric acid type A receptor tyrosine phosphorylation in the hippocampus. *Proc Natl Acad Sci USA* 106(47):20039–20044.
2. Lee HH, et al. (2007) Direct protein kinase C-dependent phosphorylation regulates the cell surface stability and activity of the potassium chloride cotransporter KCC2. *J Biol Chem* 282(41):29777–29784.
3. Lee HH, Deeb TZ, Walker JA, Davies PA, Moss SJ (2011) NMDA receptor activity downregulates KCC2 resulting in depolarizing GABAA receptor-mediated currents. *Nat Neurosci* 14(6):736–743.
4. Deeb TZ, Nakamura Y, Frost GD, Davies PA, Moss SJ (2013) Disrupted Cl(-) homeostasis contributes to reductions in the inhibitory efficacy of diazepam during hyperexcited states. *Eur J Neurosci* 38(3):2453–2467.

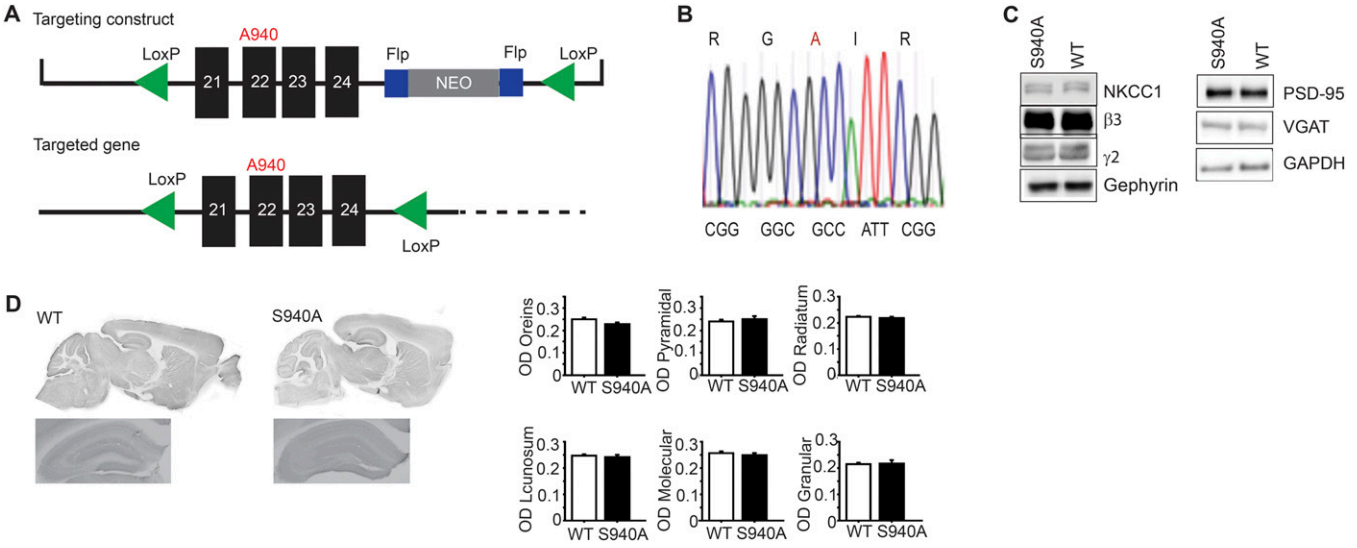

Fig. S1. Generation and characterization of S940A mice. (*A*) Diagrams of the S940A targeting construct and the successfully targeted S940A allele of KCC2. (*B*) Chromatogram indicating the presence of the S940A mutation in homozygous mice. (*C*) Hippocampal lysates from WT and S940A mice were blotted for NKCC1, $GABA_A$ receptor subunits β3 and γ2, as well as markers of excitatory and inhibitory synapses PSD-95 and VGAT, and the scaffolding protein gephyrin. (*D*) The 40-μM hippocampal slices were stained with antibodies against KCC2 followed by HRP-conjugated secondary antibody. Immunoreactivity was then visualized by diaminobenzadeine staining. Optical density analysis revealed no changes in KCC2 expression in the stratum oriens, pyramidal, radiatum, lacunosum, lacunosum moleculare, or granular regions of the hippocampus between WT and S940A mice. Values are mean ± SEM.

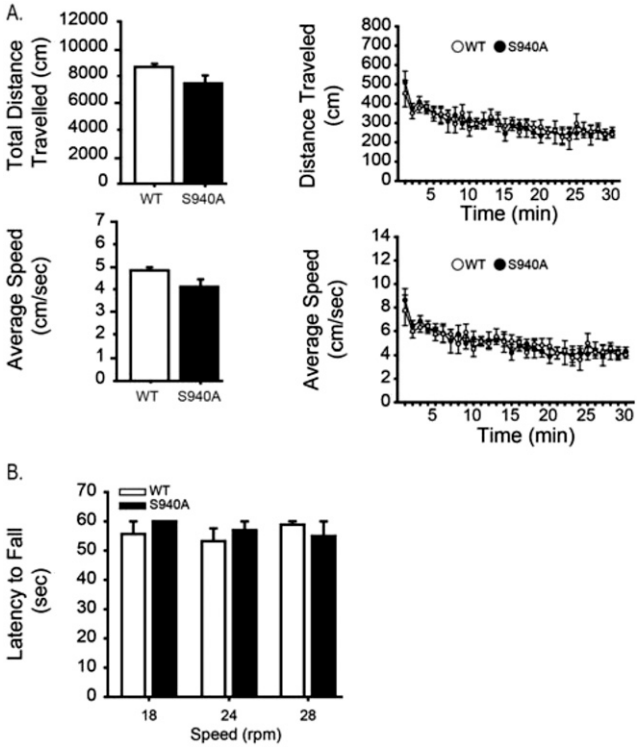

Fig. S2. Behavioral analysis of KCC2-S940A mice. (*A*) Open field travel distance and average speed do not differ between WT and S940A mice. (*B*) Rotarod latency to fall did not differ for WT and S940A mice. Values are mean ± SEM ($n = 12$ for each genotype).

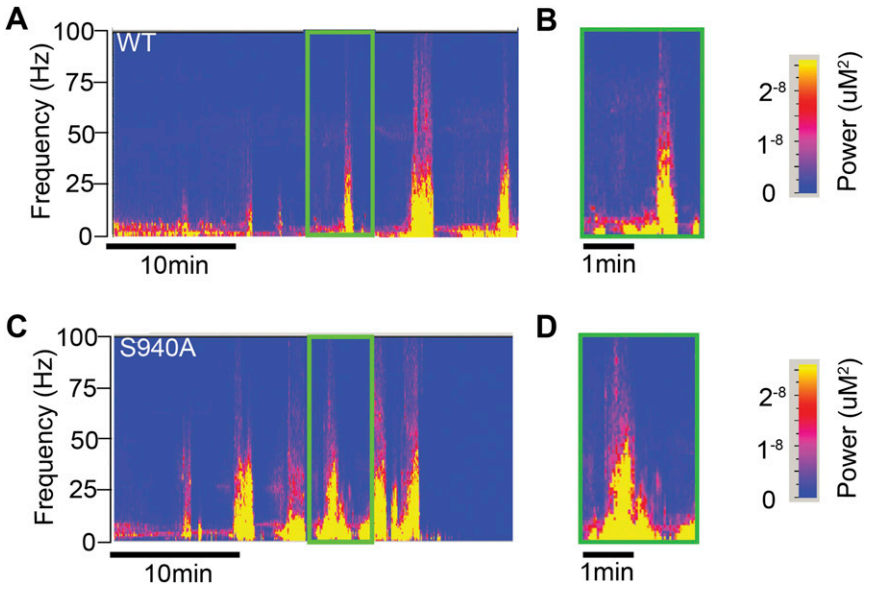

Fig. S3. Frequency power analysis of EEGs for WT and S940A mice. Power spectra of EEG data recorded during 20 min of SE are shown for WT (*A*) and S940A (*C*) mice. (*B* and *D*) Events during the 3-min time period marked by the green box in the 20-min recording.

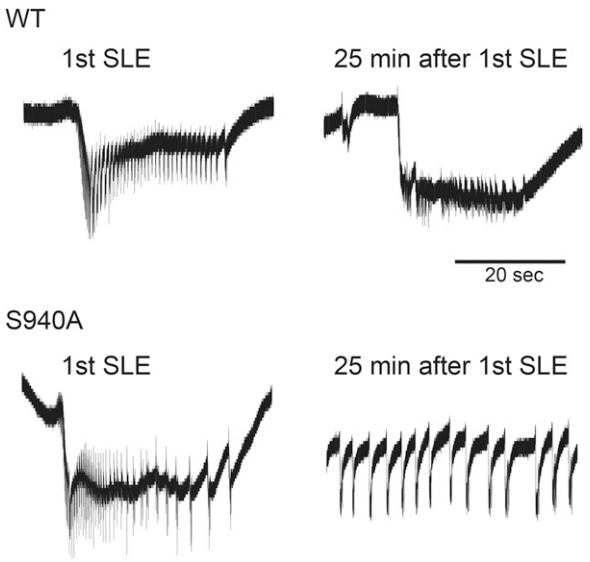

Fig. S4. Field potential recordings in entorhinal cortex revealed differences in the temporal progression of epileptiform discharges between WT and S940A slices. High-resolution traces from Fig. 2*E*. (*Left*) Exemplar traces of the first low Mg^{2+}-induced SLE for WT (*Upper*) and S940A (*Lower*) slices. (*Right*) Exemplar traces 25 min after the first SLE. The traces shown are not post hoc filtered.