Supplementary Methods

**Sanger Sequencing**

Primers were designed using Primer3 (http://frodo.wi.mit.edu/) to amplify the genomic DNA that encodes the C-terminus of KCC2 (SLC12A5) (encompassing amino acids 894–1086 [NP_065759] encoded by the nucleotides in exons 21–25 [NM_020708.4]), along with the homologous region in KCC3 (SLC12A6) (amino acids 979–1570 [NP_598408] encoded by exons 22–24 [NM_133647.1]), and the N-terminal regulatory region of NKCC1 cotransporter (SLC12A2) (amino acids 150–252 [NP_001037] encoded by c.446–756, the end of exon 1 [NM_001046]). Primer sequences are available upon request. Polymerase chain reactions (PCRs) were performed using the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California, USA) as per the manufacturer’s instruction. To visualize DNA fragments, 5 µL of the PCR product was loaded on a 1% agarose gel. Ethidium bromide was used for the staining. PCR products were sequenced at the Genome Quebec Innovation Centre (Montreal, Quebec, Canada) using a 3730XL DNA Analyzer (Applied Biosystems, Foster City, California, USA), and analyzed using the SoftGenetics program, Mutation surveyor (v.3.10, SoftGenetics, State College, Pennsylvania, USA).

**Protein Sequence Alignment and In Silico Prediction Programs**

Conservation of the KCC2 protein across species was determined by aligning the following orthologues: Homo sapiens (NP_065759), Macaca mulatta (XM_001104494.2_prot), Rattus norvegicus (NP_599190), Mus musculus (NP_065066), Takifugu rubripes (ENSTRUT0000047011) and Danio rerio (ENSDART00000009569). The effects of amino acid substitutions on protein function were predicted using MutationTaster, Panther, and SIFT.

**Statistical Analysis**

All statistical analysis for genetics was carried out using the program R (version 2.15.1). Fisher’s exact test was used to generate the p-values and odds ratios.

**Gramicidin perforated patch clamp recording**

Gramicidin perforated-patch clamp recording was performed from N2a cells transfected with a mixture of two mammalian expression constructs in a modified Clontech backbone vector harboring the human α1 subunit of the glycine receptor (GlyR), and N-terminal mCherry-tagged WT or IGE mutant KCC2. For control experiments, mCherry-KCC2 was substituted with an mCherry pcDNA construct (mock transfected cells). The transfection was performed using Lipofectamine 2000 according to manufacturer’s protocol (Life Technologies SAS). Recordings were performed 48 to 56 h after transfection. Coverslips with transfected N2a cells were placed onto the inverted microscope and perfused with an external solution (in mM): 140 NaCl, 2.5 KCl, 20 HEPES, 20 D-glucose, 2.0 CaCl₂, 2.0 MgCl₂, pH 7.4. Recordings were performed as described in [2, 3]. Briefly, the recording micropipettes (5 MΩ) were filled with a solution containing (in mM): 150 KCl, 10 HEPES, 20 mg/ml gramicidin A, pH 7.2. The tip of the pipette was filled with the same solution, but
without gramicidin. Glycine (50 µM) was dissolved in external solution and focally applied to soma and proximal dendrites through a micropipette (same as for patch clamp) connected to a Picospritzer (General Valve Corporation, pressure 5 p.s.i.) (Figure 2A). Recordings employed an Axopatch-200A amplifier and pCLAMP acquisition software (Axon Instruments) in voltage-clamp mode. Data were low-pass filtered at 2 kHz and acquired at 10 kHz. Input resistance ($R_{in}$) and capacitance were routinely determined from the analysis of responses to hyperpolarizing/depolarizing steps of $-10/+10$ mV applied from the holding potential ($V_h$) of $-60$ mV. Before measurement of $I-V$ relationships, a single test pulses was applied at $V_h=-60$ mV to determine the direction of $I_{Gly}$ current, adjust the position of the pipette, and time agonist application with the final aim being to obtain short (300-1000 ms) current responses of low amplitude (less than $-100$ pA). Depending on the direction of current recorded at $-60$ mV, four glycine responses were recorded at voltages $-120$, $-100$, $-80$, $-60$ mV (for outwardly directed [positive] glycine-induced currents at $-60$ mV) or $-80$, $-60$, $-40$ and $-20$ mV (for inwardly directed [negative] glycine-induced currents at $-60$ mV) as shown in Figure 2B.

To test the function of heterozygote-like expression of KCC2 mutants, we co-expressed constructs encoding GlyR, eGFP-KCC2 into the N2a cells [2] and mCherry KCC2 in the proportions 1.4:1.0:1.0. For recordings, cells were chosen that showed similar intensities of eGFP and mCherry fluorescence by CCD camera acquisition and Metamorph software. The ranges of the fluorescence were identically set for all mutants.

**Assay of KCC2 surface expression**

In order to directly visualize the insertion of KCC2 IGE variants into the membranes of single neurons, we created a novel construct encoding KCC2 with a fluorescent tag introduced into the second extracellular loop of the transporter (KCC2-pH$_{ext}$; details provided on request). KCC2-pH$_{ext}$ was transected using a magnetofection kit after 10 days of *in vitro* primary culture of hippocampal neurons prepared from E19 rat embryos as described previously (Buerli et al., 2007). 48 h after transfection, neurons were rinsed with external solution containing in mM: 140 NaCl, 2.5 KCl, 20 HEPES, 20 D-glucose, 2.0 CaCl$_2$, 2.0 MgCl$_2$, pH 7.4 and incubated for 40 min at 18°C with polyclonal anti-GFP antibody (1:1000, Molecular Probes) dissolved in the same solution. The coverslips were rinsed x3 and fixed with Antigenfix (Diapath, Martingo, Italy) for 20 min (at RT), permeabilized with 0.3% Triton X-100, blocked by 5% goat serum, and labeled for 1h (RT) with a secondary CY3-conjugated antibody. The images were acquired with an Olympus Fluorview-500 confocal microscope (40×; 1.0 NA). Quantitative analysis of KCC2-pH$_{ext}$ surface labeling was performed using Metamorph software (Roper Scientific).

**Assay of KCC2 phosphorylation status at S940**

Detection of the phosphorylation state of S940 in cellular lysates expressing WT and IGE mutant KCC2 was performed essentially as described in detail in [4]. Briefly, HEK293 cells were cultured in DMEM with 10% FBS and 5%
penicillin/streptomycin, and transfected using the Biorad nucleofection system in OPTIMEM media per the manufacturer’s instructions. After 2 days to allow sufficient expression, cells were lysed in 1X RIPA lysis buffer supplemented with protease and phosphatase inhibitors and subjected to SDS-PAGE. Millipore α-KCC2 antibody was used to detect total KCC2, and a phospho-specific antibody directed against S940 [4] was utilized to detect the phosphorylation state at this residue, as previously described.

Ratiometric Cl-Sensor imaging

Ratiometric imaging of Cl-Sensor fluorescence was performed from N2a cells transfected with a mixture of three mammalian expression constructs in a modified Clontech backbone vector harboring Cl-Sensor, the human α1 subunit of the glycine receptor (GlyR), and an N-terminal mCherry-tagged WT or IGE mutant KCC2. For control experiments, mCherry-KCC2 was substituted with empty vector pcDNA3.1 (mock-transfected cells). Excitation of transfected cells was performed every 20 s through 500 (20) nm and then 430 (24) nm narrow-band excitation filters (referred to as 500 nm and 430 nm filters). Detailed sequences of constructs are available upon request; experimental procedures are provided in [3].

Three-dimensional Structure Modeling

The three-dimensional structure of human KCC2 C-terminal domain (CTD) was modeled by the I-TASSER [5] server with input KCC2 amino acid sequences 648 to 1116. Crystal structure of the cytoplasmic domain of a prokaryotic cation chloride cotransporter (PDB accession code 3G40) and several other structures were used as templates for comparative modeling. The top template 3G40 shows 26.67% sequence identity with KCC2 CTD. Among the five models predicted from the server, the one forming organized structure with a high C-score was chosen. The C-score of the modeled KCC2 CTD is -0.54, indicating high reliability and quality of the overall structural model. PyMOL was used for molecular graphics displaying.

REFERENCES
