

Research paper

Severe neonatal epilepsy linked to a novel homozygous *SLC12A5* Arg508Cys variant with impaired chloride transport

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ABSTRACT

The potassium-chloride cotransporter 2 (KCC2) is a neuron-specific transporter essential for maintaining low intracellular chloride levels. By extruding chloride ions, KCC2 ensures that activation of GABA_A receptors produces hyperpolarizing inhibitory responses rather than depolarizing responses. Disruption of KCC2 function can therefore impair GABAergic signaling and neuronal maturation, contributing to a range of neurodevelopmental and neurological disorders. Pathogenic biallelic variants in *SLC12A5*, the gene encoding KCC2, are a rare cause of severe early-onset developmental and epileptic encephalopathies, including epilepsy of infancy with migrating focal seizures (EIMFS). Here, we describe a novel homozygous *SLC12A5* variant identified in a patient with severe, drug-resistant epilepsy, neonatal encephalopathy, and rapid neurological deterioration. Combined Western blot, thallium (Tl⁺) flux, and gramicidin-perforated patch-clamp assays revealed significantly reduced ion-transport function of the KCC2 construct encoding the variant, with no change in protein expression abundance or profile. Live-cell surface immunolabeling demonstrated markedly reduced plasma membrane expression and decreased internalization of the variant, suggesting that the functional deficit primarily results from defective trafficking or reduced membrane stability. These findings expand the spectrum of KCC2-related disorders and highlight the critical role of KCC2 in early brain development. By linking a specific *SLC12A5* variant to impaired chloride homeostasis and neuronal hyperexcitability, this study provides mechanistic insight into disease pathogenesis and lays the groundwork for therapeutic strategies aimed at restoring or stabilizing KCC2 function.

1. Introduction

The potassium-chloride cotransporter 2 (KCC2), encoded by *SLC12A5*, is a neuron-specific transporter that extrudes chloride ions from mature neurons. By maintaining low intracellular chloride

concentrations ([Cl⁻]_i), KCC2 ensures that activation of GABA_A receptors (GABA_AR) drives chloride influx, producing effective synaptic inhibition (Ben-Ari, 2002; Owens and Kriegstein, 2002). Through this regulation of [Cl⁻]_i, KCC2 determines whether γ -aminobutyric acid (GABA) functions as an inhibitory or excitatory neurotransmitter in the

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central nervous system (CNS) (Schmidt et al., 2018; Bormann et al., 1987; Chamma et al., 2012; Amadeo et al., 2018).

In immature neurons, intracellular chloride concentration remains high due to the predominant activity of the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter NKCC1, which imports Cl^- using the Na^+ gradient maintained by the Na^+/K^+ -ATPase (Yamada et al., 2004; Tillman and Zhang, 2019). This elevated $[\text{Cl}^-]_i$ depolarizes the GABA reversal potential (E_{GABA}), making GABA_AR activation excitatory rather than inhibitory. During development, upregulation of KCC2 expression gradually lowers $[\text{Cl}^-]_i$ by extruding Cl^- in symport with K^+ , utilizing the K^+ gradient maintained by the Na^+/K^+ -ATPase to drive Cl^- efflux. This activity hyperpolarizes E_{GABA} and converts GABAergic responses from depolarizing to hyperpolarizing. This mechanism establishes the inhibitory role of GABA and contributes to the balance between neuronal excitation and inhibition (Zhang et al., 2006; Rivera et al., 1999; Plotkin et al., 1997).

Impaired KCC2 function disrupts chloride homeostasis, elevating $[\text{Cl}^-]_i$ and reducing GABA_AR-mediated inhibition, thus predisposing neurons to hyperexcitability and seizure activity (Cohen et al., 2002; Huberfeld et al., 2007). Disease-associated *SLC12A5* variants have been reported across a spectrum of neurodevelopmental and neuropsychiatric disorders. Heterozygous variants often showed incomplete penetrance and were associated with relatively mild epilepsy phenotypes, including febrile seizures (FS) and idiopathic generalized epilepsy (IGE) (Puskarjov et al., 2014; Kahle et al., 2014; Hübner, 2014). Biallelic variants were linked to severe epileptic encephalopathies, particularly epilepsy of infancy with migrating focal seizures (EIMFS) (Stöberg et al., 2015; Järvelä et al., 2024; Saito et al., 2017; Saito et al., 2016; Hamze et al., n.d.). Additional associations have been reported with autism spectrum disorder (ASD) and schizophrenia (SZ) (Merner et al., 2015). Most reported KCC2 mutations were single amino acid substitutions distributed across multiple functional domains, highlighting the importance of structural integrity for transporter function (Stöberg et al., 2015; Järvelä et al., 2024; Saito et al., 2016; Hamze et al., n.d.).

In this study, we described a novel homozygous *SLC12A5* variant (c.1522C > T; p.Arg508Cys) in a patient presenting with severe neonatal epileptic encephalopathy. The functional impact of this variant was assessed in heterologous in vitro expression systems using gramicidin-perforated patch-clamp recordings and thallium flux assays. These experiments revealed a substantially impaired capacity for chloride extrusion. Structural modeling further suggested a destabilizing effect of the Arg508Cys substitution on the KCC2 protein, consistent with impaired membrane stability. In addition, surface immunolabeling further indicated a substantial decrease in the membrane localization of the mutant KCC2. Collectively, these findings demonstrated that the *SLC12A5* variant causes a loss of function, leading to disrupted chloride homeostasis and impaired GABAergic signaling, and provide a mechanistic basis for the pathogenicity of the variant.

2. Materials and methods

2.1. Genetic testing and data analysis

Genomic DNA was extracted from peripheral blood. Whole-exome sequencing was performed using the Twist Library Preparation EF kit (Twist Bioscience), enabling analysis of coding regions and exon-intron boundaries of approximately 20,000 protein-coding genes. Sequencing was performed on an Illumina NextSeq platform, achieving 98.7% of the targeted regions with ≥ 20 -fold coverage and a mean coverage of 111-fold. The *SLC12A5* gene was covered at 98.9% with > 20 -fold coverage and an average of 80-fold. Analysis included a virtual epilepsy gene panel (panels CsNd, 2023), a metabolic disease gene panel (Genetics B, 2023). Variants were cross-referenced with the ClinVar (v2024-01-07) and HGMD Professional 2023.4 databases, and phenotype-genotype associations were evaluated using the Franklin variant classification algorithm. Variants with a minor allele frequency (MAF) below 1% in population databases and located in phenotype-associated genes were

prioritized for analysis. The identified variant was subsequently confirmed by Sanger sequencing. Written informed consent was obtained from the patient's parents, and the study was conducted in accordance with the principles of the Declaration of Helsinki.

2.2. Cell culture

Human embryonic kidney (HEK293) and mouse neuroblastoma (N2a) cells were obtained from ATCC (CRL-1573TM and CCL-131, respectively) and cultured on coverslips in 24-well plates or in 3.5 cm Petri dishes (gramicidin-perforated patch-clamp). The cells were maintained in a 1:1 mixture of minimal essential medium (MEM or Eagle's MEM; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany; for gramicidin-perforated patch-clamp experiments) and Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The medium was supplemented with 8–10% fetal bovine serum (FBS or FBS Good; PAN-Biotech GmbH, Aidenbach, Germany; for gramicidin-perforated patch-clamp experiments), 100 IU/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Cytiva-HyClone Laboratories Inc., Logan, UT, USA), and the cells were incubated under a 5% CO_2 atmosphere at 37 °C. N2a cells, up to passage 10, were utilized for perforated patch-clamp experiments, while cells up to passage 15 were employed for immunolabeling.

2.3. Plasmid constructs

Plasmid expression vectors encoding the human KCC2b splice variant (NM_020708.4) have been described previously (Kahle et al., 2014; Järvelä et al., 2024). Three constructs were used: non-tagged KCC2, KCC2 fused to an N-terminal mCherry tag (mCherry-KCC2), and KCC2 with an extracellular pHluorin tag (KCC2-pH_{ext}). The $\Delta\text{N-KCC2-pH}_{\text{ext}}$ construct, which lacks the N-terminus and is unable to localize to the cell surface, served as a negative control in surface immunolabeling (Friedel et al., 2017). Site-directed mutagenesis was performed to introduce the variant into the KCC2 constructs using the QuikChange II kit (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. The mutagenesis primer sequences are presented in Supplementary Table S1. Mutant plasmids were transformed into StellarTM competent cells (Takara Bio, Saint-Germain-en-Laye, France, Cat. No. 636763) for propagation. The presence of the desired nucleotide substitution was confirmed by Sanger sequencing using 12 primers (Table S2).

For co-transfections in perforated patch-clamp, a Clontech vector (Clontech Laboratories Inc./Takara Bio USA Inc.) containing the human glycine receptor $\alpha 1$ subunit was used. The plasmid construct encoding the fluorescent protein mCherry, pCAGImC_Empty, was a gift from Joshua Mendell (Addgene plasmid #92015; <http://n2t.net/addgene:92015>; RRID: Addgene_92015; (Golden et al., 2017)).

2.4. Transient transfection

HEK293 and N2a cells were seeded 24 h prior to transfection. HEK293 cells were transfected with TurboFect (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Transfection efficiency, assessed by immunocytochemistry, routinely reached 20–30%. Transfected HEK293 cells were used for K^+/Cl^- cotransporter activity measurements using a thallium flux assay 24 h post-transfection. N2a cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) in combination with CombiMag Magnetofection Reagent (OZ Biosciences, Marseille, France) for surface immunolabeling, or Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific) for gramicidin-perforated patch-clamp recordings. Transfected N2a cells were used for immunolabeling and electrophysiological recordings 48–72 h post-transfection.

2.5. Immunocytochemistry

HEK293 cells grown on poly-L-lysine-coated coverslips were fixed with 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4) for 10 min at room temperature (RT), then washed three times with PBS. Blocking was performed for 30 min using 2% BSA and 10% goat serum in PBS. Cells were incubated for 1 h with primary antibodies (anti-KCC2 N1–12, 1:1000; Neuromab; Invitrogen) diluted in carrier solution (0.3% Triton X-100, 1% BSA, 1% goat serum in PBS). After washing, secondary antibodies (Alexa Fluor 488 goat anti-mouse, 1:1000; Thermo Fisher Scientific) were applied for 1 h in the dark. Cells were washed, air-dried, and mounted with Mowiol containing DAPI (1:1000; Roth). Images were acquired using an Olympus BX63 fluorescence microscope.

2.6. Western blot analysis

Transfected HEK293 cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (w/v) Triton X-100, 0.27 M sucrose, 10 mM dithiothreitol (DTT), and protease inhibitor cocktail (Thermo Scientific, Waltham, MA, USA; Cat#78425). Lysates were clarified by centrifugation at 14,000 $\times$ g for 15 min at 4 °C. The cleared lysates were mixed with 2 $\times$ Laemmli sample buffer (Bio-Rad, Hercules, CA, USA; Cat#161–0747) and incubated at 37 °C for 15 min. Proteins were separated by SDS–PAGE using 4–15% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad; Cat#4561083) and subsequently transferred onto nitrocellulose membranes for immunoblot analysis.

Overexpressed KCC2 was detected using a rabbit anti-KCC2 polyclonal primary antibody (K0120–07, 1:5000; USBiological), followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:10,000; Bio-Rad; Cat#170–6515). β -Actin was used as a loading control and detected using an anti- β -actin antibody (sc-47778, 1:5000; Origene). Detected bands were visualized using the ECL Prime Chemiluminescence kit (GE Healthcare, Chicago, IL, USA). Images were acquired with the Azure 600 Imaging System (Azure Biosystems, Dublin, CA, USA), and exposure times were optimized to maintain signal intensities within the linear detection range and avoid saturation. Relative intensities were quantified by densitometric analysis using ImageJ software.

2.7. Thallium-based KCC2 flux measurements

KCC2-mediated transport activity was assessed by measuring Cl^- -dependent TI^+ uptake in HEK293 cells, using a fluorescence-based assay described previously (Hartmann et al., 2010). Cells were cultured in 96-well plates and, prior to measurement, the growth medium was replaced with 80 μL of hypotonic preincubation buffer (100 mM *N*-methyl-D-glucamine chloride, 5 mM HEPES, 5 mM KCl, 2 mM CaCl_2 , 0.8 mM MgSO_4 , 5 mM glucose; pH 7.4; osmolarity: 175 mmol/kg $\pm$ 2) containing 2 μM FluoZin-2 AM dye (Invitrogen) and 0.2% (w/v) Pluronic F-127. Cells were incubated for 48 min at RT in the dark to allow for dye loading. After incubation, cells were washed three times with preincubation buffer and then treated with 80 μL of the same buffer containing 0.1 mM ouabain for 15 min at RT to inhibit Na^+/K^+ -ATPase activity. The plate was subsequently transferred to a fluorometer (Fluoroskan FL, Thermo Fisher Scientific), and 40 μL of 5 $\times$ thallium stimulation buffer (12 mM Ti_2SO_4 , 100 mM *N*-methyl-D-glucamine, 5 mM HEPES, 2 mM KCl, 2 mM CaCl_2 , 0.8 mM MgSO_4 , 5 mM glucose; pH 7.4) was injected into each well. Fluorescence was recorded kinetically (excitation 485 nm, emission 538 nm) at 6-s intervals for 200 s. Transport activity was determined from the initial slope of TI^+ -stimulated fluorescence, normalized to KCC2^{WT} (100%) to account for baseline fluctuations.

2.8. Gramicidin perforated patch-clamp experiments

To complement the thallium flux assay in functional assessment of KCC2, we performed gramicidin-perforated patch-clamp experiments, in which glycine receptor (GlyR) mediated Cl^- current through the cellular membrane was recorded similarly as described previously (Järvelä et al., 2024). Transiently co-transfected N2a cells cultured on uncoated glass coverslips were placed in a recording chamber and submerged in a bathing solution containing (in mM) 140 NaCl, 2.5 KCl, 20 D-glucose, 10 HEPES, 2 CaCl_2 , 2 MgCl_2 , and 0.01 Bumetanide. pH of the bathing solution was adjusted to 7.4 with NaOH. Osmolality of the bathing solution ranged from 305 to 315 mOsm/kg. 3.5–6 M Ω microelectrodes were pulled from borosilicate capillaries with filament and outer and inner dimensions of 1.5 and 0.86 mm, respectively (Warner Instruments, Harvard Apparatus, Holliston, MA, USA), with the P-87 Flaming/Brown Micropipette Puller (Sutter Instrument, Novato, CA, USA). Microelectrodes were filled with a pipette solution containing (in mM) 150 KCl, 10 HEPES, and 20 $\mu\text{g}/\text{mL}$ gramicidin-D. Gramicidin-D was added to the pipette solution just prior to experiments from a stock solution of 20 mg/mL gramicidin-D dissolved in DMSO. pH of the pipette solution was adjusted to 7.2 with KOH, and the osmolality of the pipette solution, with gramicidin added, varied in the range of 290–300 mOsm/kg. The liquid junction potential (LJP) for the solutions used was estimated to be 4.5 mV. A glycine stock solution was prepared by diluting glycine to dH_2O to 100 mM. Glycine was added to the extracellular solution to a final concentration of 100 μM . All chemicals were acquired from Sigma-Aldrich (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) unless stated otherwise. Cells were visualized using an upright microscope Nikon Eclipse FN1 (Nikon Europe B.V., Amstelveen, The Netherlands), with a 40 $\times$ water-immersion objective and an epifluorescence system. Successfully transfected cells were identified by fluorescence.

Recordings were proceeded with once a giga-seal was successfully formed, and the access resistance (R_a) had dropped to less than 120 M Ω due to gramicidin perforation. Median R_a was 52.5 (33.8, 72.5) M Ω with all cells combined ($n = 28$). In three cells, the 100 M Ω limit was exceeded, reaching maximally 120 M Ω in one cell. Cells were discarded during recordings or excluded from the analysis if the input resistance dropped below 350 M Ω (763.5 (587.4, 1227.2) M Ω , $n = 28$) or if the cell showed strong instability or baseline drift. Subsequently, a voltage-clamp protocol consisting of test voltage steps (between –100 and –20 mV) from a holding potential of –60 mV with 10 mV increments was applied. During each voltage step, glycine was focally and transiently applied to the patched cell with a micropipette connected to a PLI-100 Plus Pico-Injector (5 psi; Harvard Apparatus, Holliston, MA, USA). The largest Cl^- current peaks produced were kept <400 pA by adjusting the positioning of the Pico-Injector micropipette for each recorded cell to avoid Cl^- build-up and to minimize voltage drops due to uncompensated series R_a . The voltage steps range was adjusted to elicit a sufficient number of GlyR-mediated Cl^- current responses below and above the reversal potential of the current (E_{Gly}), which is largely determined by Cl^- concentrations. Micropipettes were manipulated with Sensapex SMX series micromanipulators (Sensapex, Oulu, Finland). Recordings were performed at a room temperature of 21 °C.

Recordings were performed using a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA, USA), digitized with Axon Digidata 1550B data acquisition system (Molecular Devices, San Jose, CA, USA), and data collected with pCLAMP 10.7 data acquisition software (Molecular Devices, San Jose, CA, USA). Recordings were sampled at 10 kHz and filtered using the 4-pole Bessel filter of the Multiclamp 700B at a cutoff frequency of 2 kHz. Data were analyzed with Clampfit 10 (Molecular Devices, San Jose, CA, USA), Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), and OriginPro 2025 (OriginLab Corporation, Northampton, MA, USA).

2.9. Surface immunolabeling

N2a cells were transiently transfected with constructs containing an external pHluorin tag, KCC2^{WT}-pH_{ext}, KCC2^{R508C}-pH_{ext}, ΔN-rKCC2-pH_{ext}, using a combination of Lipofectamine 2000 and CombiMag Transfection Reagent, following the manufacturer's protocols. Forty-eight hours after transfection, cells were incubated at 37 °C in 5% CO₂ for 1 h with a polyclonal rabbit anti-GFP antibody (SAB4701015, Sigma-Aldrich, Darmstadt, Germany), diluted 1:250 in culture medium, to label the extracellular pHluorin tag. Thereafter, cells were gently washed twice with fresh culture medium. For the third wash, the culture media was replaced with HBS precooled to 13 °C. Thereafter, Alexa Fluor 647-conjugated anti-rabbit secondary antibody (1:250, A-21244, Invitrogen), diluted in HBS, was applied and cells were placed in a thermo-insulated chamber at 13 °C and incubated for 30 min. This step decorates surface-localized KCC2-pH_{ext} and creates a fluorescence pool corresponding to membrane-localized KCC2 (F_m). After incubation, the cells were gently washed twice with HEPES-buffered saline at 13 °C, then fixed in paraformaldehyde (PFA) at 4 °C for 30 min. The fixed cells were washed with PBS at RT, then incubated for 1 h at RT in a PBS solution containing 0.3 M glycine, 0.3% Triton X-100, and 5% goat serum to permeabilize the plasma membrane and block nonspecific antibody binding. The fixed and permeabilized cells were incubated at RT for 1 h with a goat anti-rabbit Alexa 555-conjugated antibody (1:400, A-21428, Invitrogen) to visualize the internalized molecules previously labeled with the anti-GFP antibody (F_i). Subsequently, the cells were incubated with chicken anti-GFP antibody (1:500, 1020, Aves Labs). This was followed by staining with Alexa FluorTM 488-conjugated goat anti-chicken antibody (1:1,000, A-11039, Invitrogen) to detect the total amount of KCC2-pH_{ext} overexpressed in the cells (F_t). All antibody solutions, both primary and secondary, were centrifuged at 8,000 g for 5 min to remove any debris before use. To quantify the amount of internalized KCC2-pH_{ext} (F_i, internalized fluorescence pool), we analyzed only Alexa 555-positive molecules, excluding those co-labeled with Alexa 647.

Images of labeled cells were captured using an Olympus Fluoview-500 confocal microscope equipped with a 40× oil-immersion objective, a zoom level of 3, and an image resolution of 1024 × 1024 pixels. Each z-stack comprised 9 optical sections, with each plane having a thickness of 1 μm and spaced 1.5 μm apart. Transfected cells were randomly selected based on Alexa Fluor 488 fluorescence. Z-stack images were acquired to capture the emissions of Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647. Fluorescence signals were detected using the green (excitation: 488 nm, emission: 505–525 nm), red (excitation: 543 nm, emission: 560–600 nm), and far-red (excitation: 633 nm, emission: 660 nm) channels, respectively. The fluorescence intensities of labeled KCC2-pH_{ext} of each cell were collected with MetaMorph software. For statistical analysis of F_i, F_m, and F_t, we used absolute fluorescence values and normalized each of them to the corresponding KCC2^{WT}-pH_{ext} median to enhance data visualization.

2.10. Structural modeling

Secondary structure predictions were performed using Jpred4 and GOR4 (Drozdetskiy et al., 2015; Garnier et al., 1996). The effect of the Arg508Cys mutation on KCC2b protein stability was analyzed using the I-Mutant 2.0 (Capriotti et al., 2005) and DynaMut (Rodrigues et al., 2018) online tools, with the 6M23.pdb and 7D8Z.pdb structures used as input (Berman et al., 2000; Chi et al., 2021a). The structure of KCC2 embedded in a lipid bilayer was visualized using coordinates available in the MemProtMD database (Newport et al., 2019). Structural analyses were based on the canonical human sequences of KCC1 (PDB ID: 7AIP) (Chi et al., 2021b), KCC2b (PDB ID: 6M23), KCC3 (PDB ID: 7D90), and KCC4 (PDB ID: 7D99) (Xie et al., 2020). Structural coordinates were obtained from the RCSB Protein Data Bank (Berman et al., 2000). Sequence alignments and structural representations were generated

using ESPript 3.0 (Robert and Gouet, 2014).

3. Results

3.1. Clinical description of the patient

The patient was a newborn when admitted to the Perinatal Intensive Care Unit (PICU) of the Department of Pediatrics at the Clinical Center, University of Debrecen, Hungary. The patient was the third child and third daughter of healthy parents (Fig. 1A); her mother had experienced seizure episodes during childhood. One of the patient's older sisters experienced five febrile seizure episodes in her lifetime. Pregnancy was uncomplicated. The patient was born at term with normal birthweight (10th–25th percentile) and Apgar scores of 5/1, 6/5, and 8/10. Immediately after birth, the patient exhibited increased muscle tone in the limbs with apparent stiffness, axial hypotonia, flexion of the upper limbs, extension of the lower limbs, trismus, and absence of cry. Crying eventually began with physical stimulation, and she required brief assisted ventilation using a Neopuff device. Unusual tonic postures, accompanied by parallel drops in oxygen saturation, began at 5 min of age. Apneic spells continued throughout, necessitating transfer to the PICU. The tonic episodes associated with apnea were resolved with intravenous midazolam. Immediately after admission to the PICU, a loading dose of intravenous phenobarbital was administered. Physical examination at admission revealed an open mouth, retrognathia, barely opening eyes, sluggish sucking and pharyngeal reflexes, a monophasic and sluggish Moro reflex, poor spontaneous movements, and limbs held in forced positions. The patient exhibited contractures of the shoulders, elbows, hips, and knees, with flexion of the upper limbs, extension of the lower limbs, and axial hypotonia. The fingers were deviated toward the ulnar side, with fingers II–IV overlapping finger III. Additionally, there was a fine tremor in the right upper limb, a 2 cm umbilical hernia, apneic episodes accompanied by apparent “tension,” and drops in oxygen saturation. Due to initial respiratory distress and feeding difficulties, the child required nasal CPAP support for five days and prolonged tube feeding. Newborn metabolic screening tests were negative. Extended laboratory tests and radiological examination were performed. Infections were excluded. The MR examination did not detect organic anomalies.

The pediatric neurological examination revealed generalized hypotonia and severe neonatal encephalopathy, which dominated the clinical presentation. Amplitude-integrated electroencephalography (aEEG) showed severe, diffuse brain dysfunction with epileptiform abnormalities (Fig. 1B–F). Both the aEEG and video EEG studies confirmed epileptiform dysfunction against the background of encephalopathy and recurrent episodes of deep apnea. The predominant seizure semiology was tonic, involving all four limbs and the axial muscles, although tonic-clonic seizures were also observed. Video EEG demonstrated severe dysfunction, with alternating-sided and diffuse spike activity, bilateral and multifocal ictal and interictal epileptiform discharges, and non-convulsive status epilepticus affecting the right, left, or both fronto-centro-temporal regions. A combination of alternating antiepileptic agents, including phenobarbital, levetiracetam, phenytoin, lamotrigine, clonazepam, pyridoxine, and valproic acid, was administered. No significant change in seizure frequency or electroclinical features was observed, and seizure freedom was not achieved with treatment. Cytogenetic testing confirmed a normal karyotype (46, XX), and whole-exome sequencing was subsequently performed. Given the genetic findings and the lack of response to combined, alternating antiseizure therapy, a ketogenic diet was planned. However, before it could be initiated, the patient experienced a sudden, profound drop in oxygen saturation accompanied by malaise, followed by bradycardia and asystole. Despite advanced cardiorespiratory resuscitation, the patient died at 3 months of age.

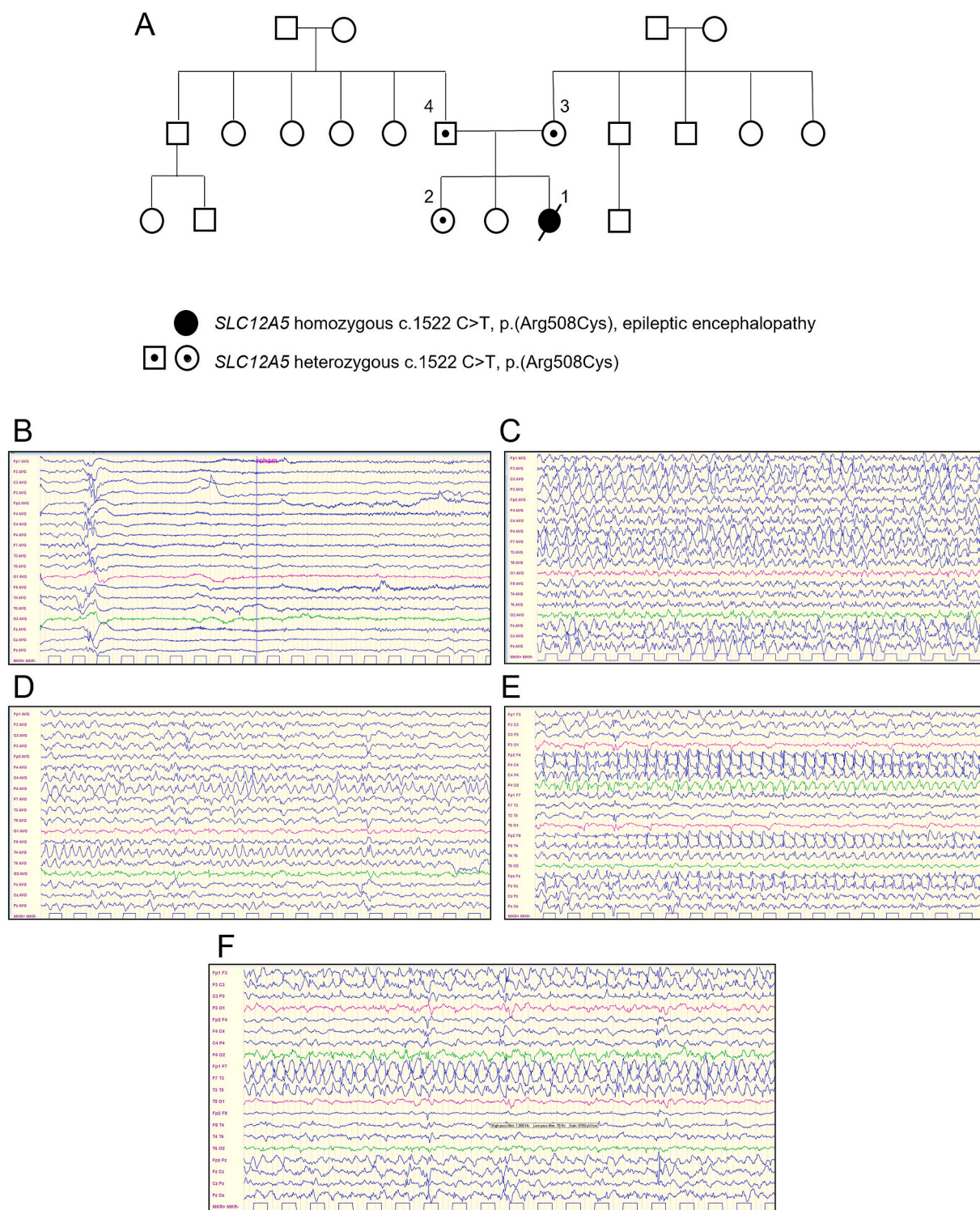


Fig. 1. Family pedigree and EEG findings of the patient carrying the *SLC12A5* c.1522C > T (p.Arg508Cys) missense variant. A. Family pedigree showing that the proband (individual 1) is homozygous for the c.1522C > T (p.Arg508Cys) variant and died at 3 months of age. The proband's 5-year-old sister (individual 2), who experienced five episodes of febrile seizures, is heterozygous for the variant. The proband's mother (individual 3), with a history of childhood epilepsy, is also heterozygous. The proband's father (individual 4) is asymptomatic but heterozygous; his brother also had childhood epilepsy (not available for testing). Lower panel, Electroencephalography (EEG) examination. B. At the age of 2 months, the patient showed a tonic seizure with an ictal pattern (vertical line indicates the start of seizure). C. During the EEG examination, the patient showed the same tonic tightening. During the seizure, apnea was recognized, and she required oxygen supplementation. D. Severe diffuse brain dysfunction. 4–5 Hz rhythmic theta activity over the right and left hemispheres. E, F. It shows spike activity, with most spike waves occurring at 2–2.5 Hz. The recordings indicate right-left (bilateral) fronto-centro-temporal non-convulsive status epilepticus.

3.2. Results of the whole-exome sequencing analysis

The variant was identified as NG_046341.2: g.28409C > T (hg19), a single-nucleotide change resulting in transcript-specific consequences: NM_001134771.2: c.1591C > T (p.Arg531Cys) in the KCC2a isoform and NM_020708.5: c.1522C > T (p.Arg508Cys) in the KCC2b isoform. The result of whole-exome sequencing for the patient is provided in Fig. S1. This variant has not been previously described in the literature and is listed once in the ClinVar 2024–06 database as a variant of uncertain significance (VUS; ID: 2582468). Parental testing confirmed that both parents are heterozygous carriers, supporting the patient's homozygous state (Fig. 1A). The older sister who experienced febrile seizures was also found to be heterozygous for this variant (Fig. S1). Based on ACMG guidelines, this variant can be reclassified as likely pathogenic. The PS3 criterion is supported by functional data demonstrating a loss-of-function effect on KCC2 activity (see below). The patient's phenotype, developmental and epileptic encephalopathy 34 (MIM #616645), is consistent with previously reported *SLC12A5*-associated cases (McTague and Kurian, 2019). The variant fulfills PM2 due to its extremely low frequency in gnomAD (3 heterozygous carriers, no homozygotes; MAF: 0.000001863). PP3 is supported by in silico tools predicting a deleterious effect, and PP2 applies, given the gene's intolerance to benign missense variation and the established role of missense mutations in disease mechanisms.

3.3. Structural insights into KCC2

KCC2 comprises an intracellular N-terminal domain (NTD), twelve transmembrane segments (TMs), a prominent extracellular loop between TM5 and TM6, and a cytoplasmic C-terminal domain (CTD) (Chew et al., 2021). Two splice variants, KCC2a and KCC2b, are generated via alternative promoter usage (Uvarov et al., 2007). High-resolution cryo-electron microscopy (Cryo-EM) revealed that the Arg508 residue of KCC2b (corresponding to Arg531 in KCC2a) is located within the TM8 helix (Fig. 2A, B) (Chi et al., 2021a; Xie et al., 2020; McMoneagle et al., 2024). The KCC2b N-terminal Ala66-Asn83 loop (KCC2a: Ala89-Asn105) functions as an autoinhibitory region that limits

access to intracellular ion-binding sites (Xie et al., 2020). Arg508 may support this state through H-bonding with Pro72 in the L12 loop (Fig. 2C), stabilizing interactions with TM1a, TM6b, and TM8. The binding of L12 to the cytosolic vestibule is characteristic of the KCC2 autoinhibitory state (Xie et al., 2020).

The predictions of the Arg508Cys variant on the protein structure indicated that the mutant KCC2 protein preserves the secondary structural arrangement of the wild-type protein, both at the variant site and in its surrounding environment. Stability analysis using the I-Mutant 2.0 web server indicated a decrease in free energy upon the Arg508Cys variant, with a calculated change of -1.0 kcal/mol for both the 6M23.pdb and 7D8Z.pdb structures used as input (Berman et al., 2000; Chi et al., 2021a), suggesting a moderate destabilizing effect. Complementary structure-based predictions using DynaMut yielded mixed results: a stabilizing change of $+1.216$ kcal/mol for 6M23.pdb and a destabilizing change of -1.080 kcal/mol for 7D8Z.pdb (Fig. 2D). Despite this difference, the destabilizing values obtained from both I-Mutant and DynaMut were nearly identical, supporting the reliability of the predictions and indicating an overall destabilizing nature of the Arg508Cys variant. Nevertheless, the stability analysis implied that the vibrational entropy might change not only in the close proximity of the mutated position. It also predicted increased rigidity for the cytosolic domain (Fig. 2D), indicating potential distant effects of the mutation. However, the computational methods used are not sufficiently accurate to definitively predict long-distance structural changes. Overall, the Arg508Cys variant is predicted to have a moderate destabilizing effect without altering the protein's secondary structure.

The conservation of Arg508 was investigated by structural alignment of KCC1-KCC4 proteins (Fig. 3A). Comparison of crystal structures revealed that the arginine residue is highly conserved in its spatial position across these proteins. In KCC2b, Arg508 forms a hydrogen bond with the main-chain oxygen of Pro72 in the L12 loop. A similar interaction occurs in KCC4, where Arg528 forms a hydrogen bond with the side chain of Ser96 at the equivalent position (Fig. 3B). This interaction was not observed in the crystal structures of KCC1 and KCC3, as the N-terminal regions are disordered and not resolved. However, structures predicted by AlphaFold (Varadi et al., 2024) suggest that an

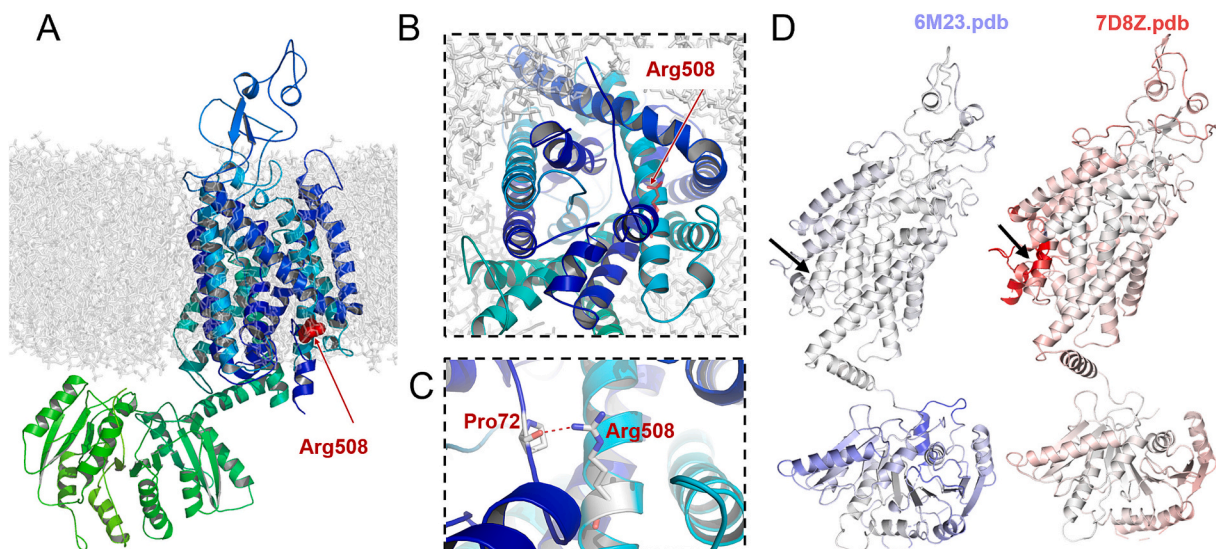


Fig. 2. Structural visualization of the Arg508 residue of the KCC2 protein. A. Structure of KCC2 (PDB ID: 6M23) embedded into a lipid bilayer. Side view of the protein is shown. The protein is colored blue, except for the C-terminal cytosolic domain, which is shown in green. B. Bottom view of the transmembrane domain; the Arg508 residue is shown as a red stick. C. Enlarged view of Arg508 residue interacting with Pro72 of the N-terminal autoinhibitory loop. D. Structural representation of the Arg508Cys KCC2 mutant generated using the DynaMut web server; residues are colored according to the vibrational entropy change upon the point mutation. Blue indicates rigidification of the structure (based on the 6M23 PDB structure), while red indicates increased flexibility (based on the 7D8Z PDB structure) (Berman et al., 2000; Chi et al., 2021a). The arrows indicate the site of the point mutation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

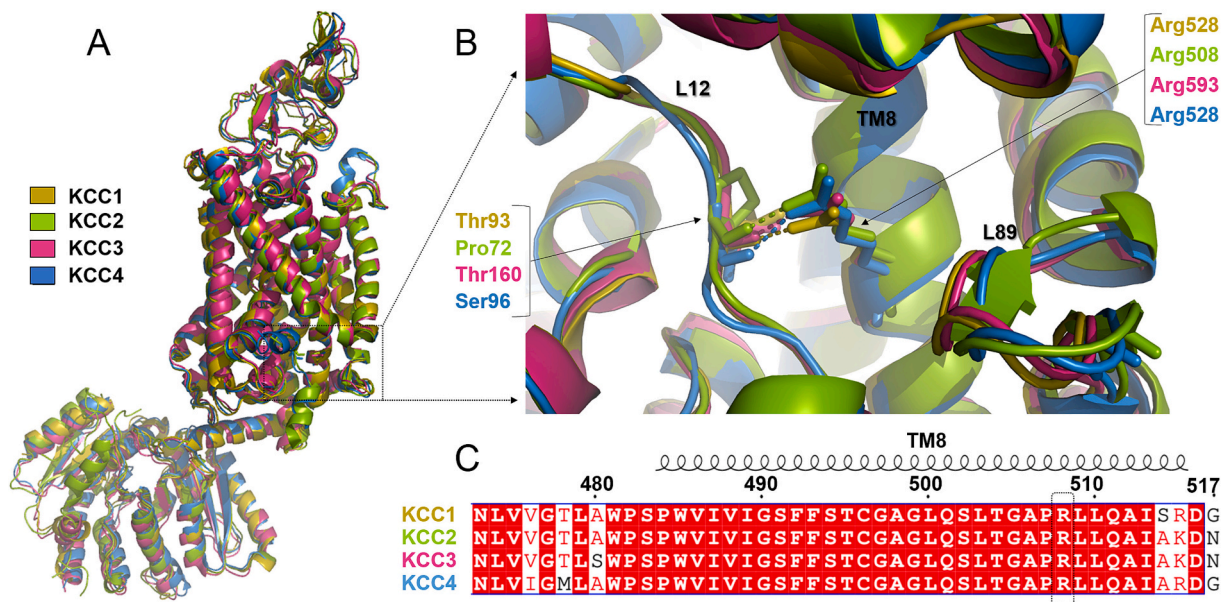


Fig. 3. Sequence and structure alignment of KCC1, KCC2, KCC3, and KCC4 proteins. A. All structures correspond to the canonical human sequences: KCC1, KCC2b, KCC3, and KCC4. Each protein chain has a different colour, as indicated in the figure. B. Enlarged view of the region encompassing the Arg508 residue and its interaction with the Pro72 residue of the N-terminal region of KCC2b. The residues of KCC1, KCC3, and KCC4 located at structurally equivalent positions are also shown. The structures of KCC2 and KCC4 are based on crystal structures, whereas those of KCC1 and KCC3 were predicted by AlphaFold. C. Sequence alignment of the TM8 helix region containing Arg508.

autoinhibitory conformation might potentially exist in KCC1 and KCC3 as well. Interactions were predicted between the main-chain oxygen of Thr and the side-chain atoms of the corresponding arginines: Thr93-Arg528 in KCC1 and Thr160-Arg593 in KCC3 (Fig. 3B). Sequence alignment revealed that the arginine residue is highly conserved across KCC1-KCC4 proteins (Fig. 3C), consistent with previous findings (Xie et al., 2020). Nearly the entire TM8 helix sequence (Trp504-Ile536, KCC2b numbering) is identical across all four KCC isoforms, highlighting the highly conserved nature of this region, including the arginine residue.

3.4. KCC2 variant for *in vitro* study

To directly assess whether the single amino acid Arg508Cys substitution alters the properties of KCC2, we generated cDNA constructs encoding the non-mutated (wild-type, WT) human KCC2b isoform (KCC2^{WT}) as well as KCC2^{WT}-derived constructs harboring the Arg508Cys substitution (KCC2^{R508C}).

Western blot analysis of the KCC2^{R508C} variant overexpressed in HEK293 cells revealed a multiband migration pattern similar to that of KCC2^{WT} (Kok et al., 2024; Agez et al., 2017), including a ~ 120 and 140 kDa double bands corresponding to monomeric forms and > 250 kDa bands corresponding to oligomeric forms (Fig. 4A). The overall abundance of overexpressed KCC2^{R508C} protein, including both monomeric and oligomeric forms, was comparable to that of KCC2^{WT}, with no significant difference between the two conditions (Fig. 4B). In contrast, the monomeric fraction of KCC2^{R508C} was consistently and significantly reduced relative to KCC2^{WT} across all analyzed samples (Fig. 4C). Together, these findings indicate that the mutant protein is correctly translated and expressed at levels similar to those of the wild-type transporter, while also suggesting altered structural organization favoring oligomerization of the KCC2^{R508C} complex. The mechanisms underlying this shift in oligomerization remain unclear (Kok et al., 2024; Medina et al., 2014). The analysis of the ratio of glycosylated to total monomeric KCC2 revealed no difference between KCC2^{R508C} and KCC2^{WT}, indicating that the Arg508Cys substitution did not impair transporter maturation in the endoplasmic reticulum (ER) (Fig. 4D).

3.5. Thallium assay of KCC2's ion-transport activity

To evaluate the functionality of KCC2 harboring the Arg508Cys variant (KCC2^{R508C}), a thallium (Tl⁺) influx assay, an indirect measure of K⁺-Cl⁻ cotransporter activity, was performed (Hartmann et al., 2010). HEK293 cells were transiently transfected with human non-tagged KCC2^{WT} and KCC2^{R508C} constructs. Immunocytochemistry results showed that the transfection efficiency of cells expressing KCC2^{R508C} was comparable to that of cells expressing KCC2^{WT}, indicating that the variant does not affect transfection efficiency (Fig. 5A, B). Fluorescence kinetics upon Tl⁺ application were recorded in HEK293 cells expressing KCC2^{WT}, KCC2^{R508C}, or mock construct under control conditions and in the presence of the KCC2 inhibitor VU0463271. The signal was reduced in KCC2^{R508C} compared to KCC2^{WT}, while mock-transfected cells exhibited minimal fluorescence changes over time (Fig. 5C). Transport activity was calculated from the slope of the fluorescence signal following Tl⁺ application. Quantitative analysis of Tl⁺ flux intensity under control conditions revealed that KCC2^{R508C} diminished ion-transport activity by more than twofold compared to KCC2^{WT}. Despite this reduction, KCC2^{R508C} exhibited significantly higher transport activity than Mock-transfected cells, suggesting that the variant retains partial functional capacity (Fig. 5D). Furthermore, the strong reduction of the fluorescence signal in the presence of the KCC2 inhibitor VU0463271 confirms that the measured Tl⁺ influx predominantly reflects KCC2-mediated transport activity, supporting the specificity and validity of the assay (Fig. 5D).

3.6. Gramicidin perforated patch-clamp experiments

To further verify the functional consequences of the Arg508Cys substitution on KCC2-mediated chloride extrusion while preserving physiological intracellular chloride concentrations, we performed gramicidin-perforated patch-clamp recordings of GlyR-mediated Cl⁻ currents in transiently co-transfected N2a cells. Cells expressed the GlyR α 1-subunit together with either mCherry-tagged KCC2^{WT}, KCC2^{R508C}, or mCherry alone as a mock. By plotting GlyR-mediated peak Cl⁻ currents (Fig. 6A) against step voltages, E_{Gly} values could be obtained (Fig. 6B).

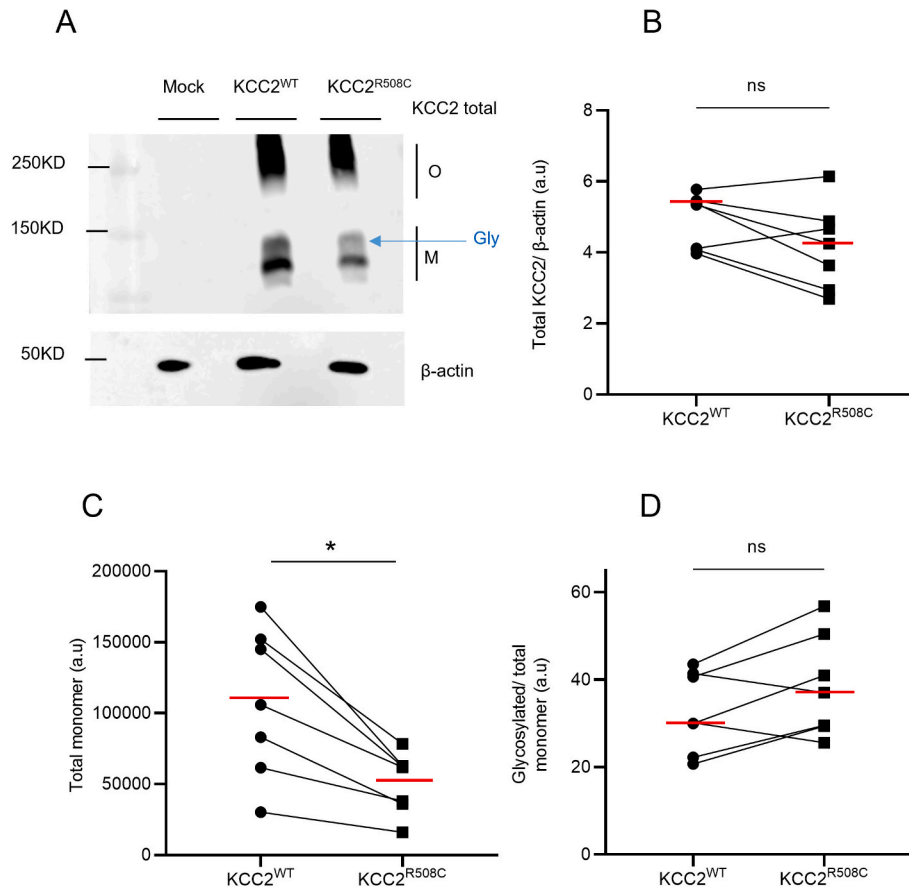


Fig. 4. Western blot analysis of KCC2^{R508C} variant properties. A. Western blot analysis of protein expression in HEK293 cells transfected with Mock, KCC2^{WT} and KCC2^{R508C} constructs. Full-length blots are shown in Fig. S3. Oligomeric and monomeric forms are indicated by O and M, respectively. The glycosylated band is indicated by “Gly” and the arrow. The lower panel shows β-actin immunoreactivity on the same membrane used for KCC2 detection. B. Paired quantitative immunoblot analysis of total KCC2 normalized to β-actin. Individual paired values are connected by lines. Red lines denote median values. C. Paired quantitative immunoblot analysis of total KCC2 monomers. D. Paired quantitative immunoblot analysis of the ratio of glycosylated to total KCC2 monomers. Statistical analysis was performed using the Wilcoxon matched-pairs test. Data represent at least four independent experiments ($n = 7$). * $P < 0.05$; ns, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As E_{Gly} in our recordings reflects the steady-state $[\text{Cl}^-]_i$, it can be used for a coarse assessment of the efficacy of Cl^- extrusion. Quantitative analysis showed a statistically significant 17.8 mV depolarizing shift in E_{Gly} for KCC2^{R508C} compared with its wild-type counterpart (Fig. 6C), indicating higher $[\text{Cl}^-]_i$ in variant-transfected cells. For mock-transfected cells, this shift was even higher, with the difference between KCC2^{WT} and mock median values being 23.6 mV. However, the median E_{Gly} value for KCC2^{R508C} was slightly more hyperpolarized than that of the mock, with a difference of 5.8 mV, but this difference was not statistically significant. Thus, gramicidin-perforated patch-clamp results indicate impaired Cl^- extrusion in cells expressing KCC2^{R508C}.

3.7. Cell surface expression of Arg508Cys variant

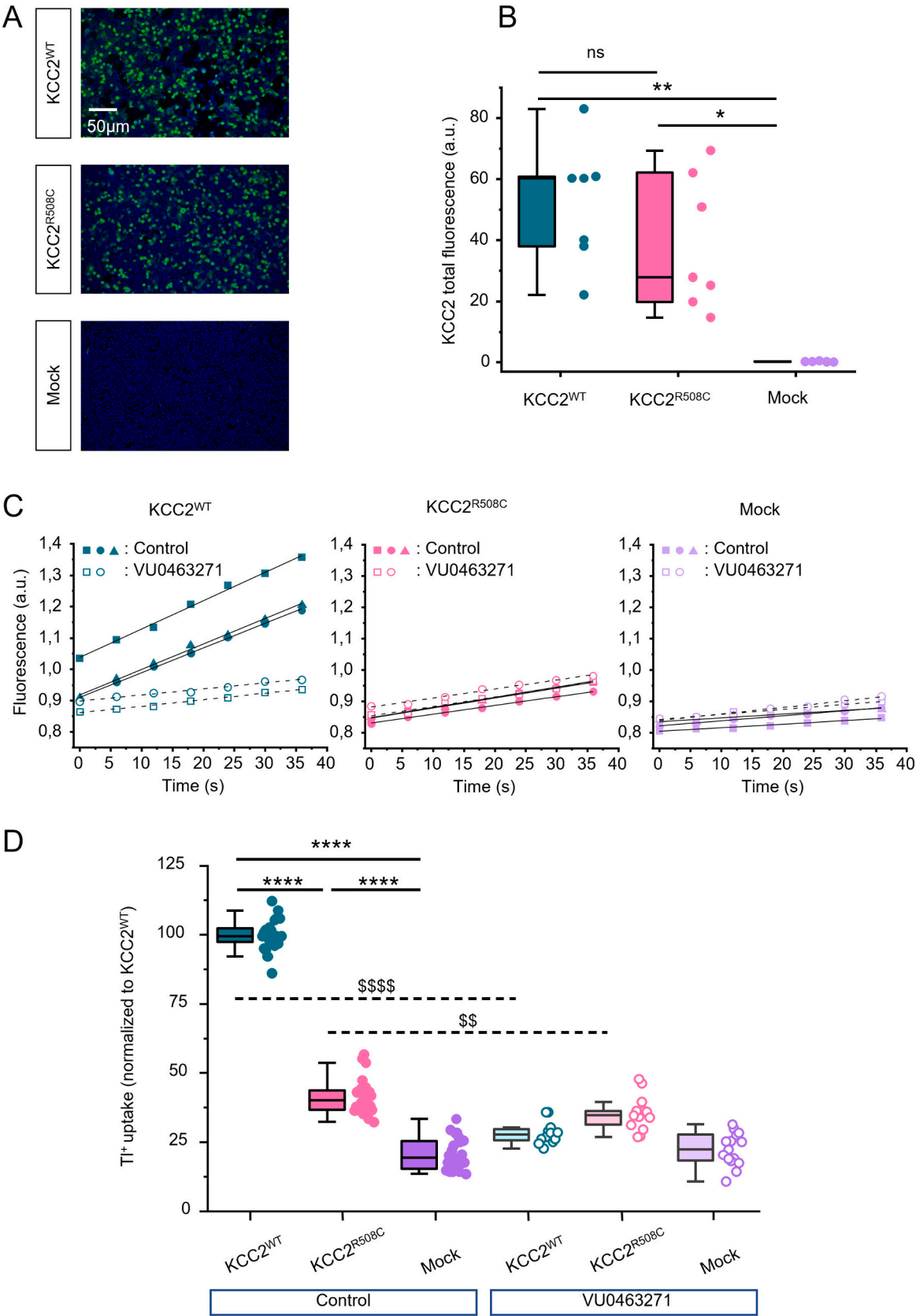
To evaluate the surface expression of the KCC2, we employed a previously developed multi-step live-cell immunolabeling strategy using KCC2-pH_{ext} (Fig. 7A) (Friedel et al., 2017). Consistent with previous reports (Järvelä et al., 2024; Friedel et al., 2017), the labelling of cells expressing KCC2^{WT}-pH_{ext} produced images with well detectable pools of plasma membrane localized molecules (F_m) at the perimeter of the cell and internalized molecules (F_i) distributed throughout the cytoplasm, with increased density in the perinuclear area (Fig. 7B). In contrast to wild-type KCC2, the majority of cells expressing the KCC2^{R508C}-pH_{ext} mutant were decorated with a markedly lower number of cell-surface-localized fluorophores, and exhibited substantially reduced fluorescence intensity associated with internalized molecules. Analysis

revealed a significant more than twofold decrease in the median value of F_m and a fivefold reduction in F_i (Fig. 7C). It is unlikely that the observed reduction in F_m was due to compromised expression of KCC2^{R508C}-pH_{ext} in the analyzed cells, as the total amount of KCC2^{R508C}-pH_{ext} protein (F_t) was not significantly different from that measured in KCC2^{WT}-pH_{ext} expressing cells. Consistent with this, analysis of the F_m -to- F_t ratio revealed a threefold difference between cells expressing KCC2^{WT}-pH_{ext} and KCC2^{R508C}-pH_{ext} (Fig. S2).

4. Discussion

This study reported a novel case of severe early infantile developmental and epileptic encephalopathy associated with a homozygous c.1522C > T (p.Arg508Cys) variant in *SLC12A5*, which encodes the neuron-specific K^+/Cl^- cotransporter KCC2. The affected individual exhibited very early-onset, highly refractory epilepsy and neonatal encephalopathy, progressing to marked neurological deterioration and early mortality. Functional characterization of the identified variant revealed reduced KCC2 activity, leading to disrupted chloride homeostasis and impaired GABAergic signaling, which likely represents the underlying pathogenic mechanism.

To date, only two homozygous missense variants in *SLC12A5*, Lys288His and Arg231His, have been functionally examined in patients with epilepsy of infancy with migrating focal seizures (EIMFS), while the remaining variants are compound heterozygous (Puskasjov et al., 2014; Kahle et al., 2014; Stöckberg et al., 2015; Saito et al., 2017; Saito et al.,



(caption on next page)

Fig. 5. Ti^+ flux measurements in HEK293 cells transiently transfected with KCC2^{WT} and $\text{KCC2}^{\text{R508C}}$. A. Immunocytochemistry was used to monitor the transfection rate of cells expressing KCC2^{WT} and $\text{KCC2}^{\text{R508C}}$ (green), and cells were counterstained with DAPI (blue). B. Quantitative analysis of the total intensity of KCC2-related fluorescence across the entire optical field. In boxplots, boxes span the interquartile range (Q1–Q3), the line inside indicates the median, and whiskers define the outermost data point that falls within upper inner and lower inner quartile fences [$Q1-1.5(IQR)$] and [$Q3-1.5(IQR)$], respectively. $N = 7$ for KCC2^{WT} and $\text{KCC2}^{\text{R508C}}$, and $N = 5$ for mock-transfected cells. Kruskal–Wallis test followed by Dunn's post hoc test: KCC2^{WT} vs $\text{KCC2}^{\text{R508C}}$, $P = 1.0$ (ns, not significant); KCC2^{WT} vs Mock, $P = 0.00401$ (**); $\text{KCC2}^{\text{R508C}}$ vs Mock, $P = 0.03159$ (*). C. Examples of the fluorescence kinetics upon application of Ti^+ to cells expressing the indicated constructs under control conditions (vehicle) and in the presence of the selective KCC2 inhibitor VU0463271 (10 μM , 15 min pre-incubation). The slope of the fluorescence increase was used as a measure of Ti^+ influx. D. Boxplots of the normalized Ti^+ flux intensity under control conditions and in the presence of VU0463271. Asterisks indicate the difference between KCC2^{WT} and $\text{KCC2}^{\text{R508C}}$ variants revealed using the Kruskal–Wallis test followed by Dunn's post hoc test: **** $P < 0.0001$. Dollar signs (\$) indicate differences between cells expressing the same constructs but assayed in the presence or absence of 10 μM VU0463271. Mann–Whitney test: \$\$, $P < 0.01$; \$\$\$, $P < 0.0001$. For vehicle: $N = 24$ for three conditions; For VU0463271: $N = 12$ for KCC2^{WT} , $N = 13$ for $\text{KCC2}^{\text{R508C}}$, and $N = 15$ for mock-transfected cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

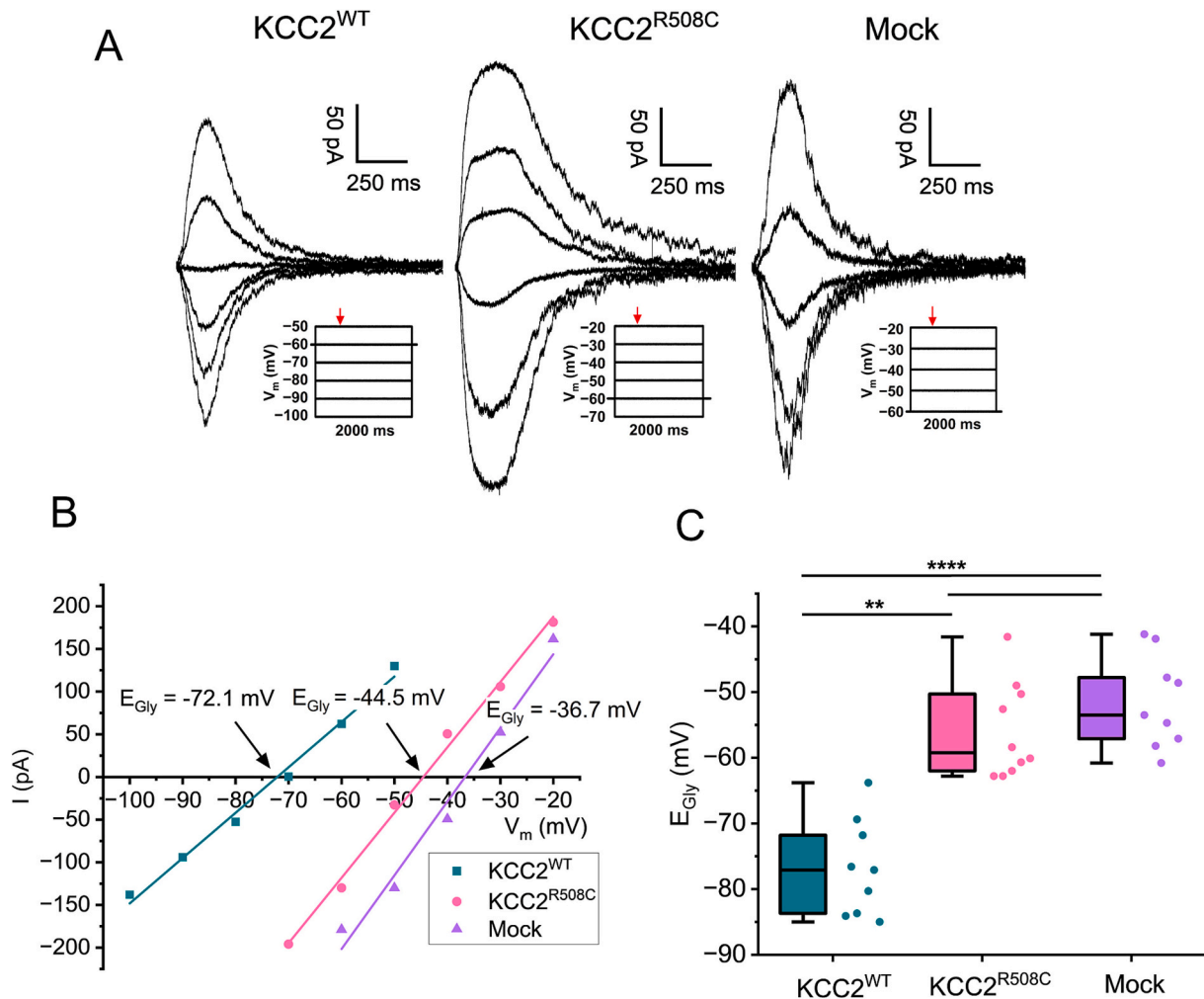


Fig. 6. Gramicidin-perforated patch-clamp on N2a cells transiently co-transfected with the $\alpha 1$ -subunit of glycine receptor in conjunction with human mCherry-tagged KCC2^{WT} , $\text{KCC2}^{\text{R508C}}$, or mCherry. A. Examples of current traces recorded from different conditions: KCC2^{WT} , mCherry-transfected control (mock), and $\text{KCC2}^{\text{R508C}}$. The voltage clamp protocols used to record the current traces are shown in the insets. Glycine was applied during the voltage steps at $t = 500$ ms to elicit GlyR-mediated Cl^- currents, the time point of which is shown in the protocol insets by arrows. No corrections for LJP have been done. B. I–V-relations of the singular cells in A. Peak GlyR-mediated Cl^- currents have been plotted against the clamped membrane potentials (insets in A). The arrows point to reversal potentials (E_{Gly}), where $I = 0$. No corrections for LJP have been done for the shown voltages. C. E_{Gly} of the GlyR-mediated Cl^- currents visualized as box plots with the data point distribution visible. The values have been corrected for the estimated LJP. Median E_{Gly} values (with interquartile ranges) in mV: KCC2^{WT} -77.1 (–83.9, –70.6) $n = 9$; $\text{KCC2}^{\text{R508C}}$ -59.3 (–62.2, –50.0) $n = 10$; mCherry (mock) -53.5 (–57.7, –44.9) $n = 9$. $N = 15$ experiments with 1–6 cells. Pairwise comparisons were done using Dunn's post hoc test after Kruskal–Wallis analysis of variance. ** $P < 0.01$, **** $P < 0.0001$.

2016; Hamze et al., n.d.). The Arg508Cys case showed notable genetic and phenotypic overlap with these rare homozygous presentations (Stöberg et al., 2015; Järvelä et al., 2024). Consistent with our findings, both previously described homozygous variants, p.Arg231His, located in transmembrane domain 4 (TM4), and p.Lys288His, situated in the extracellular loop between TM5 and TM6 of the KCC2b isoform, were

associated with reduced surface expression of KCC2, which is thought to represent the principal mechanism underlying transporter dysfunction (Stöberg et al., 2015; Järvelä et al., 2024). The clinical outcomes across these cases are similarly severe, with three of four affected individuals dying in early infancy.

Despite convergence on reduced surface expression, the underlying

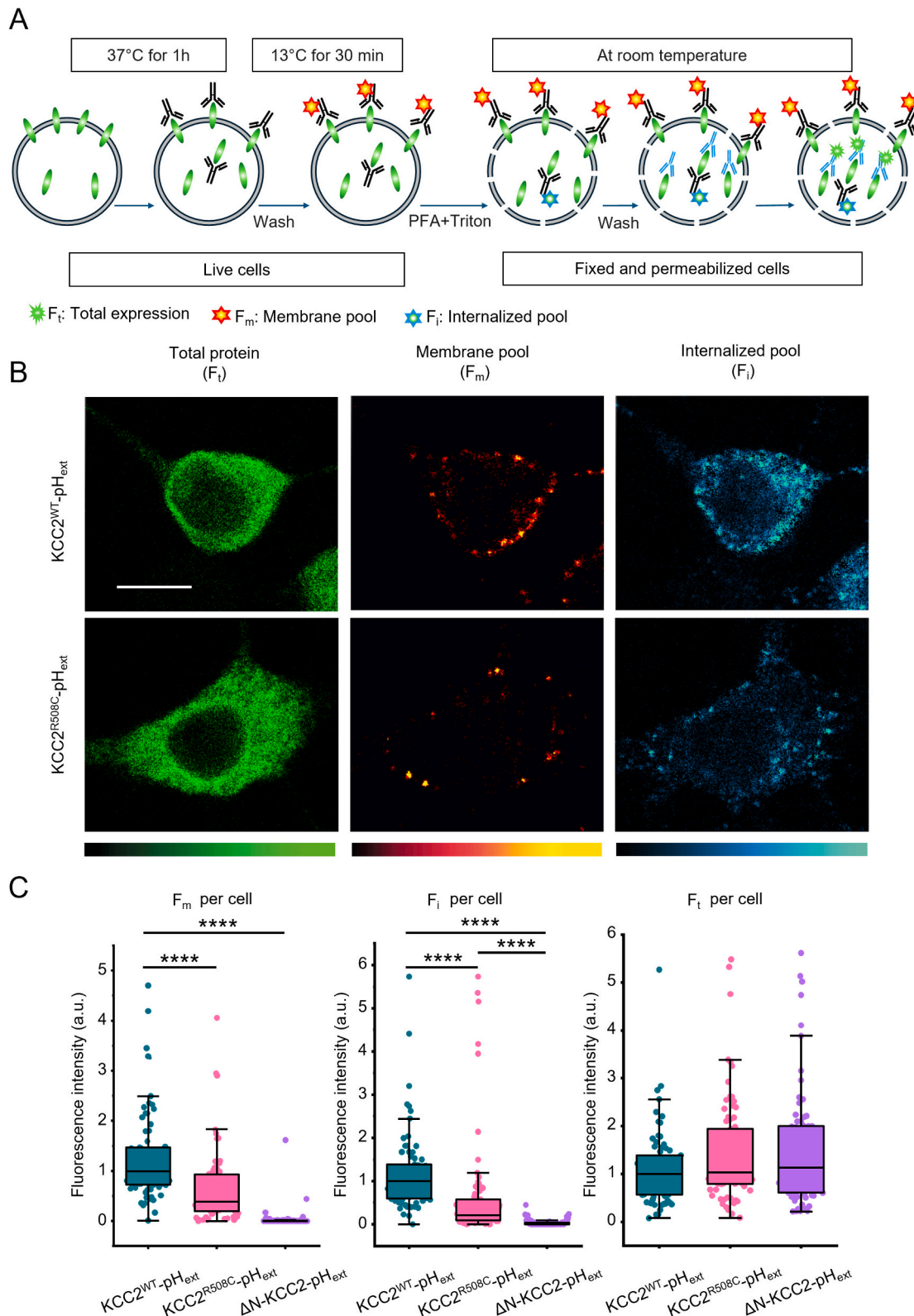


Fig. 7. Cell Surface immunolabeling of N2a cells expressing KCC2- pH_{ext} constructs. **A.** Schematic illustration of the sequential immunolabeling protocol used to detect the different pools of surface-labeled KCC2- pH_{ext} . Rods in the scheme represent the KCC2 molecules. Y are primary anti-KCC2 antibody. Asterisk illustrates the distinct pools labeled with the secondary antibody. The detailed protocol description is provided in the methods. **B.** Representative single scan confocal fluorescence images showing the F_t and F_m and F_i signals in Na2 cells expressing $KCC2^{WT-pH_{ext}}$, $KCC2^{R508C-pH_{ext}}$. Scale bar 15 μm . Colored bars at the bottom represent LUTs used for pseudocoloring to visualize fluorescence intensities. See Fig. S2A for an illustration of the assembled images used for quantification of the fluorescence intensities per cell. **C.** Quantitative analysis of F_t , F_m and F_i fluorescence intensities per cell. Data from four independent experiments ($n = 70-80$ cells per construct per experiment) were normalized to the median of $KCC2^{WT-pH_{ext}}$. See table in Fig. S2C for median values and interquartile ranges. Statistical significance was determined by a non-parametric one-way ANOVA (Kruskal-Wallis test) followed by Dunn's multiple comparison test. **** $P < 0.0001$.

mechanisms appear to differ between variants. The p.Lys288His variant was associated with markedly impaired glycosylation, likely resulting in endoplasmic reticulum retention, whereas p.Arg231His exhibited an increased rate of protein internalization (Stöberg et al., 2015; Järvelä et al., 2024). In the present study, in addition to decreased surface expression of KCC2^{R508C}, live-cell surface labeling revealed a markedly reduced number of internalized transporter molecules. Importantly, total protein levels were unchanged, indicating that the mutant protein is efficiently synthesized but fails to properly accumulate at the plasma membrane. Consistent with this, thallium influx assays demonstrated a comparable reduction in transport activity, suggesting that the functional deficit primarily reflects reduced membrane availability rather than a complete loss of intrinsic transport function. Therapeutic strategies aimed at restoring KCC2 function, promoting surface expression, or enhancing transporter activity represent a promising avenue for the treatment of these homozygous *SLC12A5* variants (Tillman and Zhang, 2019; McMoneagle et al., 2024).

This interpretation is further supported by structural modeling. Arg508 is located within the highly conserved TM8 helix, a region implicated in the formation of the intracellular vestibule and auto-inhibitory conformational state of KCC2. Cryo-EM structures indicate that Arg508 forms a hydrogen bond with residues within the N-terminal regulatory loop, thereby contributing to stabilization of the auto-inhibitory interface (Xie et al., 2020). Substitution of arginine with cysteine would abolish this interaction and may alter local conformational dynamics. Nevertheless, in silico analyses predicted only moderate destabilization of the protein and did not suggest major disruption of secondary structure or the ion translocation pathway itself. Consistent with these predictions, the residual transport activity observed experimentally indicates that the mutant transporter retains at least partial intrinsic function.

An additional notable finding was absence of the changes in glycosylation of KCC2^{R508C} protein. Since glycosylation is associated with ER processing and transporter maturation, these data suggest that early biosynthetic processing is largely preserved. The defect therefore likely occurs at later stages of trafficking, membrane insertion, stabilization, or recycling. The reduced abundance of internalized KCC2^{R508C} further argues against accelerated endocytosis as the primary mechanism and instead favors impaired forward trafficking. Although the molecular machinery regulating KCC2 endocytosis has been actively investigated (Friedel et al., 2017; Rivera et al., 2004; Lee et al., 2007; Zhao et al., 2008; Chamma et al., 2013), considerably less is known about mechanisms governing its membrane delivery and stabilization. Our findings, therefore, highlight membrane trafficking of KCC2 as an important area for future investigation.

The gramicidin-perforated patch-clamp experiments further demonstrated impaired chloride extrusion in cells expressing KCC2^{R508C}. These findings were generally consistent with the surface expression analysis and Ti^+ influx assay, both of which indicated partial loss of KCC2 function. However, whereas the Ti^+ assay demonstrated residual transport activity of KCC2^{R508C} compared with mock-transfected cells, gramicidin recordings did not reveal a statistically significant difference between KCC2^{R508C} and mock-expressing cells, despite a modestly more hyperpolarized median E_{Gly} value in KCC2^{R508C} cells (−59 mV versus −55 mV, respectively). This apparent discrepancy likely reflects fundamental methodological differences between the assays. In contrast to Ti^+ influx and surface expression analyses, which assess more precise aspects of transporter function, E_{Gly} represents an integrated physiological readout determined by the combined activity of KCC2 together with endogenous ion channels, transporters, and chloride-regulatory mechanisms contributing to steady-state intracellular chloride concentration. These additional contributors may partially compensate KCC2-dependent chloride extrusion. Therefore, although the gramicidin recordings alone did not demonstrate a statistically significant difference between KCC2^{R508C} and mock conditions, the combined results from all three complementary approaches

consistently support a hypomorphic loss-of-function effect associated with the Arg508Cys variant rather than complete abolition of transporter activity. This interpretation is biologically more plausible in the clinical context as the complete loss of KCC2 function in rodents is incompatible with postnatal survival (Hübner et al., 2001), whereas the reported *SLC12A5*-associated developmental and epileptic encephalopathies have involved severe but still only partial impairment of transporter function.

The substantial depolarizing shift in E_{Gly} observed in KCC2^{R508C}-expressing cells further suggests elevated intracellular chloride levels in neurons expressing the endogenous transporter. Such chloride accumulation would be expected to reduce the efficacy of inhibitory GABAergic and glycinergic signaling, thereby promoting pathological neuronal hyperexcitability. These findings are particularly relevant during early brain development, when precise regulation of intracellular chloride concentration is essential for the developmental transition of GABAergic signaling from depolarizing to hyperpolarizing. Impaired KCC2 activity during this critical developmental period may therefore contribute not only to seizure susceptibility and hyperexcitability, but also to the profound neonatal encephalopathy observed in the patient.

Several limitations should be acknowledged. Functional experiments were performed in heterologous expression systems that do not fully recapitulate neuronal KCC2 regulation. Recent studies demonstrated substantial differences in KCC2 processing and degradation between HEK293 cells and neurons (Kok et al., 2024), emphasizing the importance of validating these findings in more physiological systems such as induced pluripotent stem cell-derived neurons or knock-in animal models. In addition, although our data strongly support impaired membrane trafficking as the dominant mechanism, subtle alterations in transporter kinetics or conformational regulation cannot be excluded.

In conclusion, we described a novel case of a homozygous Arg508Cys variant in *SLC12A5*, expanding the spectrum of KCC2-related epileptic encephalopathies. Functional analyses demonstrated impaired chloride transport and reduced membrane expression. The severe clinical phenotype further suggested that both transport-dependent and structural roles of KCC2 may be compromised. Together, these findings underscore the essential contribution of KCC2 to early brain development and support the development of therapeutic strategies to enhance or stabilize KCC2 function.

Authors contribution

Safoura Ghalamkari: Methodology, Data curation, Software, Visualization, Writing-original draft. Viivi Järvelä: Methodology, Software, Visualization, Data curation. Anna-Maria Hartmann: Methodology, Data curation, Writing-review & editing. Esa-ville Immonen: Investigation, Project administration, Writing-review, Supervision. Igor Medina: Investigation, Methodology, Project administration, Writing-review & editing. Marko Huttula: Investigation, Project administration. Johanna Uusimaa: Investigation, Project administration. János András Mótyán: Methodology, Data curation, Writing-review. Katalin Szakszon: clinical data curation. Gabriella Merő: clinical data curation. Renáta Hudák: clinical data curation. István Balogh: Conceptualization, Supervision, Project administration, Writing-review & editing, Funding acquisition.

CRediT authorship contribution statement

Safoura Ghalamkari: Writing – original draft, Visualization, Software, Methodology, Data curation. Viivi Järvelä: Visualization, Supervision, Methodology, Data curation. Anna-Maria Hartmann: Visualization, Software, Methodology. Gabriella Merő: Methodology, Data curation. Renáta Hudák: Methodology, Data curation. János András Mótyán: Software, Methodology. Katalin Szakszon: Resources, Investigation, Data curation. Marko Huttula: Resources, Methodology, Investigation. Johanna Uusimaa: Software, Methodology, Data curation. Esa-ville Immonen: Supervision, Project

administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Igor Medina:** Writing – review & editing, Visualization, Software, Project administration, Methodology, Data curation. **Istvan Balogh:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Ethics statement

The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki.

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Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.expneurol.2026.115882>.

Data availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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