Peripheral motor neuropathy results from defective kinase regulation of the KCC3 cotransporter

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ABSTRACT

Using exome sequencing, we identified a *de novo* mutation (c.2971A>G, Thr991Ala) in *SLC12A6*, the gene encoding the K^+-Cl^- cotransporter KCC3, in an early-onset, progressive,

and severe peripheral motor neuropathy. This mutation abolished the WNK kinase-dependent inhibitory phosphorylation at this critical regulatory site, mimicking the dephosphorylated, activated species of the transporter normally induced by pathological cell swelling. KCC3-Thr991Ala mutant mice exhibited constitutive KCC3 activity and recapitulated aspects of the clinical, electrophysiological, and histopathological findings in human. We conclude the structure and function of the peripheral nervous system depends on finely-tuned, kinase-regulated KCC3 activity, and suggest that abnormal homeostasis of cell volume may contribute to axonal degeneration in humans.

INTRODUCTION

Inherited peripheral neuropathies are heterogenous, involving at least 75 different loci (*1-3*), and are classified in part by whether the affected gene product involves the myelin sheath, axon, or both in sensory and/or motor neurons. Consideration of both the gene mutation and the resulting pathological and clinical phenotype is required to develop an appropriate diagnosis. This classification scheme is complicated because different mutations in the same gene can yield distinct disease phenotypes. For example, dominant gain-of-function (GOF) duplications in *PMP22*, encoding peripheral myelin protein 22, and recessive loss-of-function (LOF) mutations in *PMP22,* cause Charcot-Marie-Tooth disease 1A (CMT1A) and Hereditary Neuropathy with liability of Pressure Palsies (HNPP), respectively (*4*). CMT1A is characterized by distal muscle weakness with atrophy, sensory loss, slow nerve conduction velocity, and foot deformities. HNPP is a slowly progressing pressure-induced demyelinating neuropathy causing mild symptoms such as numbness and pain to more severe muscle atrophy and paralysis of affected areas.

The *SLC12A* gene family encodes K^+-Cl^- cotransporters (KCCs), electroneutral transporters that utilize favorable outwardly-directed K^+ gradients generated by the Na^+/K^+ ATPase to mediate the regulated extrusion of K^+ and Cl^- ions from cells. As such, the KCCs play important roles in cell volume homeostasis, epithelial transport, and neuronal excitability (*5*). KCC3 is encoded by *SLC12A6* and is espressed in neurons and glial cells of the central nervous system (CNS) (*6-8*), and in the primary sensory (that is, the dorsal root ganglion [DRG]) and motor nerves of the peripheral nervous system (PNS) (*6, 9*). The function and regulation of KCC3 in the nervous system is not well understood.

Autosomal recessive homozygous or compound heterozygous LOF mutations in *SLC12A6,* encoding KCC3, cause the Mendelian disease Agenesis of the Corpus Callosum with Peripheral Neuropathy (ACCPN; OMIM #218000) (*7, 10, 11*). ACCPN patients and KCC3-knockout (KO) mice exhibit severe peripheral nerve degeneration (*9, 12-15*); however, ACCPN patients also exhibit severe brain phenotypes, including improper development of the corpus callosum, hydrocephalus, developmental delay, mental retardation, and seizures (*12, 13*). GOF mutations in

KCC3 have not been identified in any organism, and the clinical consequences of overactive KCC3 in the CNS and PNS are not known.

Here, we describe a child with a severe and progressive peripheral motor neuron neuropathy with normal brain structure and function. The patient was found to have a *de novo* mutation (Thr991Ala) in KCC3, an important regulatory site of KCC3 activity for cell volume homeostasis (*16*-*18*). We show in patient cells and *in vivo* in mouse that KCC3 Thr991Ala mutation abolishes the WNK kinase-dependent inhibitory phosphorylation at this site, constituively mimicking the dephosphorylated, activated species of the transporter normally induced by cell swelling. These observations advance our understanding of KCC3 in human physiology, reveal a critical dependence of peripheral motor neurons on kinase-regulated KCC3 activity, and suggest that impaired cell-volume homeostasis may contribute to the pathogenesis of some peripheral motor neuropathies.

RESULTS

A 10-year-old boy presented to the Neuromuscular and Neurogenetic Disorders of Childhood Section at the National Institutes of Health for diagnostic evaluation of an early-onset and progressive motor-predominant axonal neuropathy. At 9 months of age, the patient first exhibited foot-dragging while crawling. At 15 months, he developed bilateral foot drop and experienced frequent falls when walking. He had no early delays in fine motor skill acquisition; but, at 3 years of age, he developed progressive proximal and distal leg weakness followed by weakness of the hand. At 8 years, he required an assistive device to ambulate and had lost independent ambulation by 9 years. The patient reported no numbness or tingling, hearing problems, learning difficulty, or seizures. Cognitive development was normal. Family history was non-contributory.

On examination there were no dysmorphic features (including high arched palate, abnormal distance between eyes, or syndactyly), and he had normal cognition, hearing, and language. There were no clinical signs of spasticity. He had pronounced muscle atrophy in his gastrocnemius, quadriceps, and hamstrings, as well as in his intrinsic hand muscles, biceps, and triceps. He had severe weakness in a distal > proximal distribution, with near lack of anti-gravity strength (scoring 2 out of a possible 5) with attempted wrist extension, finger extension and spread, and scoring 1 to 2 oout of a possible 5 in strength in all lower extremity muscle groups. Deep tendon reflexes were absent. Vibration sense, joint position sense, and pinprick testing were normal.

At 6 years, nerve and muscle biopsies were performed at the Virginia Commonwealth University Children's Hospital. The nerve biopsy showed two nerve fascicles with mild loss of myelinated axons. A neurofilament stain also indicated mild axonal loss, whereas Luxol Fast Blue and Periodic Acid Schiff stains indicated intact myelination. No onion bulbs were detected. Hematozylin & eosin stained sections of muscle showed scattered hypertrophic and numerous atrophic fibers, indicative of denervation. Adipose tissue and endomysial connective tissue were both focally increased. There was no evidence of regeneration or degeneration, inflammation, vasculitis, or perifascicular atrophy.

Magnetic resonance imaging (MRI) of the brain revealed a normal brain, including an intact corpus callosum (red arrow), brain stem, cortical folding pattern, and cerebellum (**Figure 1A and 1B**). Comparison of muscle by ultrasound analysis between the patient and a healthy age- and gender-matched child revealed increased echogenicity (an indication of adipose or fibrous tissue), reduced bulk, and prominent atrophy (Fig. 1C and D). Nerve conduction studies were compatible with a progressive motor axonal neuropathy with secondary demyelinating features (**Table 1**). At 7 years, the tibial, median, and ulnar motor nerve studies all showed a marked reduction in amplitude with moderate slowing; the facial motor nerve was also reduced in amplitude. Median and ulnar sensory responses showed mildly reduced amplitudes with mild slowing; the sural sensory response was normal. At 9 years, the peroneal motor response was absent, and median and ulnar motor studies showed a further reduction in amplitude. The sural sensory response showed mild slowing, and the median and ulnar sensory responses showed a further mild reduction in amplitude and mild slowing of conduction velocity. Blood counts were normal. The mean corpuscular volume and osmotic fragility of red blood cells (RBCs) was normal. Blood pressure and serum and urine electrolytes were normal (T**able 2**).

Genetic testing before referral to our center included a negative complete Charcot-Marie-Tooth (CMT) panel (including *PMP22* deletion and duplication testing and sequencing, and *CX32*, *MPZ*, *PMP22*, *EGR2*, *NFL*, *PRX*, *GDAP1*, *LITAF*, *MFN2*, *SH3TC2* sequencing), and negative targeted sequencing for other relevant disease-causing genes, including *SETX*, *HSBP8*, *HSPB1*, *GARS*, *BSCL2*, *ATP7A*, *TRPV4* and *IGHMBP2*. Clinical exome sequencing on DNA extracted from blood from the patient and parents was performed at Emory Genetics using the Agilent V5Plus targeted sequence capture method and IlluminaHiSeq 2000 sequencing instruments. Variants were analyzed using bioinformatics analysis through the Emory Genetics Laboratory, with a "Neuropathy Boost option" to ensure complete coverage of genes known to cause neuropathy-related disease.

A heterozygous c.2971A>G (Thr991Ala) mutation in exon 22 of *SLC12A6*, the gene encoding the KCC3 cotransporter, was identified (reference NM_133647.1), and confirmed by Sanger sequencing (**Figure 2A**). Parental segregation testing was negative, demonstrating that the mutation was *de novo*. This mutation has not been previously reported and was not represented

in any of the major databases of genetic mutations, including dbSNP, NHLBI EVS, ExAC Browser, or GEM (*24*). Thr991 is conserved among all KCCs and across evolution (**Figure 2B, C**).

Thr991 in the KCC3 cytoplasmic C-terminus is a critcal a regulatory domain of transporter activity (*25*). To predict the functional effect of this mutation, we generated a structural model of the human KCC3 C-terminal domain (Fig. 2D) using the cytoplasmic domain of a prokaryotic cation chloride cotransporter (PDB ID: 3G40) as a template. Model of Ala substitution at this site is predicted to have deleterious effects on protein function by MutationTaster (*26*), SIFT (*27*), and PolyPhen-2 (*28*).

Given the importance of Thr991 phosphorylation for swelling-regulated changes in KCC3 activity (*16, 17*), we assessed the phosphorylation and swelling-regulated function of KCC3 in patient and parental (control) fibroblasts. As positive controls, we expressed wild-type KCC3 (KCC3-WT) or KCC3-Thr991Ala in HEK293 cells (**Figure 3A**). In all conditions tested, the Thr991Ala mutation abolished KCC3 Thr991 phosphorylation, as assessed with an antibody that specifically recognizes KCC3 phosphorylated at this residue (*17*). Note that total KCC3, NKCC1, SPAK, OSR1, and ERK1 signals were unchanged, whereas phosphorylated SPAK and NKCC1 signals were significantly increased with the hypotonic low Cl^- solution, confirming that the cells responded positively to this treatment. Relative to parental fibroblasts, patient fibroblasts showed ~50% less phosphorylation at Thr991 (repeated measures one-way ANOVA: $F(3, 4) = 14.54$, $P = 0.0129$) (**Figure 3B, C**), consistent with the heterozygous status of the KCC3 Thr991Ala mutation.

To confirm that the mutation altered KCC3 function, we assessed K-Cl cotransport activity by measuring ^{86}Rb uptake under various conditions in HEK293 cells transfected either with wild-type or KCC3-Thr991Ala cDNA, or in human fibrobasts isolated from control individuals or the KCC3-Thr991Ala patient. To isolate the KCC-mediated flux, we exposed the cells to inhibitors of the Na^+/K^+ pump and NKCC1, transporters that mediate the bulk (85%) of total K^+ uptake in these cells. Conditions of WNK/SPAK inhibiton (STOCK1S-50699) activate the K-Cl cotransporter (*21, 22*). Both untransfected and wild-type KCC3 transfected HEK293 cells

showed a significant increase in K^+ uptake upon WNK/SPAK inhibition (repeated measures two-way ANOVA: $F(15, 40) = 38.43$, $P < 0.0001$), consistent with increased KCC3 activity. Furthermore, KCC3-Thr991Ala transfected cells exhibited significantly higher activity than wild-type KCC3 in all condition ($F(5, 40) = 813.9$, $P < 0.0001$) with little to no further activation with kinase inhibition (**Figure 4A).** In fibroblasts isolated from the patient, a greater furosemide-sensitive K^+ flux (difference between flux measured in the absence and presence of furosemide) was demonstrated under hypotonic low Cl^- conditions, confirming stimulation of the cotransporter (**Figure 4B**). The KCC3-Thr991Ala fibroblasts have significantly higher K^+ flux ($F(5, 40) = 146.5$, $P < 0.0001$), compared with the control fibroblasts; The furosemide treated KCC3 control fibroblasts have significantly decreased K^+ flux ($F(3, 8) = 153.2$, $P < 0.0001$); The KCC3-Thr991Ala fibroblasts also sensitive to the furosemide treatment with significantly decreased K^+ flux ($F (5, 40) = 146.5$, $P < 0.0001$).

We next assessed cell volume regulation in KCC3-Thr991Ala patient fibroblasts and KCC3-WT parental control fiboblasts. Stimulation of KCC3-WT cells with hypotonic HEPES-MEM (150 mOsm/kg H2O) resulted in a ~2.3 ± 0.6 fold increase in cell volume (at a rate of 1.03 ± 0.1 % cell volume/min) (Figure 3F), which recovered to the original basal level after returning cells to the isotonic conditions, a process termed regulatory volume decrease (RVD) (PMID: 27033257). In contrast, KCC3-Thr991Ala exhibited a near absence of an acute swelling response to hypotonic stress (1.4 ± 0.1 fold, $P < 0.02$). These results demonstrate that the greater furosemide-sensitive K^+ flux in KCC3-Thr991Ala patient cells is associated with an abnormal RVD response to acute hyponic cell swelling.

Using CRISPR/cas9 gene editing, we created a mouse that reproduces the Thr991Ala mutation found in the human patient (**Figure 5A-D**). To demonstrate functional activation of K-Cl cotransport in this mouse, we isolated fibroblasts from mouse tails and measured K^+ influx in the presence and absence of furosemide. The flux was done in an isotonic solution because the cotransporter is typically inactive under these conditions. Although no furosemide-sensitive K^+ influx was detected in fibroblasts isolated from wild-type mice, we observed a significant furosemide-sensitive K^+ influx in fibroblasts from KCC3-Thr991Ala mice, consistent with the mutation resulting in a constitutively active transporter (**Figure 5E**). We subjected a cohort of

24 young (~ postnatal day 40, P40) mice, 12 wild-type and 12 KCC3-Thr991Ala heterozygote siblings, to the accelerated rotarod test to assess motor function. The mice heterozygous for the mutant allele showed a small reduction in the rotarod performance, although the data did not reach statistical significance (repeated measures two-way ANOVA: $F(1, 22) = 3.681$, $P = 0.0681$ or ns). A second cohort of 21 (~ P40) mice, 8 wild-type, 7 heterozygotes, and 7 homozygotes mice was tested and while no statistical difference was observed between wild-type and heterozygote mice, we observed significant locomotor deficit in the homozygous mice (**Figure 5F).**

To assess overall motor coordination and fine motor movements, we utilized the balance beam task. The mice first began their trials on the standard-sized square beam (12 mm). We found a significant difference in performance between wild-type mice and homozygous mice, but no difference between heterozygous mice and wild type. During the balance beam task, wild-type and heterozygous mice quickly traversed the beam with little to no hind limb paw slips while homozygous mice displayed tendency to stall and experience multiple hind limb paw slips during their crossing. To determine the robustness of this phenotype, we assessed the performance of the mice on a narrower 6 mm cross-sectional square beam. This size beam is considered a more difficult task for the mice, in particular for those that exhibit any limb deficits. On this beam, the wild-type and heterozygous mice continued to swiftly traverse with little to no paw slips. However, the homozygous Thr991Ala mice experienced greater difficulty in crossing, with intense dragging of the hind limbs, and with multiple homozygous mice unable to complete the task. A majority of the homozygous mice began traversing the beam, displaying appropriate forelimb coordination, but would immediately fall due to the dragging of their hind limbs and inability to properly place their hind limbs onto the beam in coordination with the fore limbs. Overall, we observed a significant main effect of genotype ($P < 0.0001$, $F_{(2,17)} = 71.94$), but no significant main effect of the neurological score given per trial ($P < 0.5142$, $F_{(2,34)} = 1.23$) (Figure 6A). In Tukey's post-hoc test, there was a statistically significant difference between wild type and homozygous balance beam performance ($P < 0.001$), with no significant difference between wild type and heterozygous ($P < 0.8498$).

We next utilized the wire hang grip test to assess forelimb deficits. We observed no significant difference ($P < 0.13$, $F_{(2, 16)} = 2.3$) nor interaction ($P < 0.58$, $F_{(4,32)} = 0.72$) between genotypes (Figure 6B). While the mice were allowed to hang on the wire for 60 seconds, we did observe homozygous mice clasping to the wire while thrashing about to either side to remain on the wire. They commonly used their hindlimbs and tail to secure themselves on the wire. Comparatively, both wild type and heterozygous mice would often move to either side, but mainly used their forelimbs. Thus, while the mice demonstrated no statistical differences in their latency to hang on the wire, we observed a unique behavior that might indicate forelimb weakness. To further assess forelimb and hind limb strength, we used the force grip assay, a non-invasive method that uses a bar attached to a force transducer. All of the mice were first weighted to account for variability in mass versus force. Wild-type mice weighed 27.15 ± 1.96 g, heterozygous mice weighed 31.1 ± 1.77 g, and homozygous mice weighed 22.7 g ± 0.482 g. There were no significant differences in body mass between wild-type and heterozygous ($P < 0.20$), nor wild-type and homozygous ($P < 0.16$). There was no statistically significant grip force differences between genotypes ($P > 0.05$).

We assessed the function of the nerve based on the height of the compound muscle action potentials (CMAPs) amplitudes and the length of latencies using nerve conduction studies on the dorsal caudal tail nerve and the sciatic nerve. A decrease in amplitude is representative of axonal loss, while an increease in latency is representative of demyelination. While there was a trend towards a decreased amplitude for motor function of the sciatic nerve in heterozygous and homozygous mice, it did not reach statistical significance. In contrast, we observed a significant reduction in the amplitude for motor function in tail (Figure 6C and Table 3). Conduction latencies in all three genotypes were not statistically significantly different for either the tail or sciatic nerve for sensory measurements. However, there was significant main effect of genotype ($P < 0.016$, $F_{(2,15)}= 5.47$) and a statistically significant difference between wild-type and homozygous mice for the latencies in motor tail conduction ($P < 0.02$, Table 3). Overall, for both studies of sensory and motor conduction, homozygous mice display a trend towards increased latencies compared to the wild-type and heterozygous counter parts (Table 3).

We assessed the integrity of sciatic nerves using ultrastructural analyses. Nerve fibers from wild type and homozygous Thr991Ala mice were cut transversally to observe the integrity of the myelin sheaths surrounding the axons (Figure 7A). At high magnification (Figure 7B-7C for wild-type and 7D-7E for homozygotes), there was no abnormal pathology. At lower magnification, both genotypes exhibited fibers with double rings of myelination (Figure 7C-D). While these events are normal and indicative of improper initial contact between axons and Schwann cells (*29*), they were observed more frequently in the homozygous sections. Upon closer examination, sections of homozygous Thr991Ala mice also showed breakage of the myelin sheaths enwrapping the axon (Figure 7H-7I). This degeneration seemed to be specific to the fibers showing double myelinating Schwann cells, and was not observed in fibers with single myelinated Schwann cells. We quantified the occurrence of this double myelination breakage by counting the number of degenerating Schwann cells that were present in relation to normal single or double myelinating Schwann cells. In 3 homozygous sections, we observed 8 fibers with double-myelin breakages and 67 adjacent normal fibers, thus representing 10% of the total number. Comparatively, this was observed only once in over 50 sections of wild-type sciatic nerve.

DISCUSSION

Our report presents the first human with a *de novo* GOF mutation in KCC3, which results in an early-onset, severe, and progressive motor-predominant neuropathy. The regulated phosphorylation of Thr991 and Thr1048 is a key mechanism controlling KCC3 activity *in vitro* (*16, 17*). KCC3 is inactive in isotonic conditions due to inhibitory phosphorylation at Thr991/Thr1048 (*16, 17*). Cell swelling causes protein phosphatase-1 (PP1) and PP2A-dependent rapid dephosphorylation of these sites to increase KCC3 activity and promote regulatory volume decrease (*16, 17*). Ala substitutions at these sites have been shown *in vitro* to prevent phosphorylation and cause constitutive activity (*16, 17*). Computer modeling revealed a close proximity of Thr991 to Thr1048 in KCC3 (**Figure 2D**). Our patient's KCC3 Thr991Ala mutation recapitulates *in vivo* these *in vitro* results and is first such mutation discovered in humans.

Our combined clinical, genetic, neurobehavioral, and cell physiological results implicate this *de novo* gain-of-function (GOF) mutation in KCC3 as the cause of severe axonal neuropathy in a human patient. Although we have not yet identified additional alleles in unrelated individuals with the condition, the data here, coupled with what is known about KCC3 and site Thr991, strongly suggest this mutation causes the symptoms of the disease. This conclusion is based on the findings that (i) this novel mutation is *de novo* (that is, it is not a variant represented in the patient's parents or the general population); (ii) Thr991 is evolutionarily conserved; (iii) Thr991Ala results in predicted pathogenicity on the basis of *in vitro* modeling; (iv) inhibitory phosphorylation of Thr991 is a key mechanism that regulates KCC3 activity and cell volume homeostasis (*16, 17*); (v) Thr991Ala prevents this phosphorylation and results in a GOF effect in patient fibroblasts, paralleling effects *in vitro* (**Figure 4**, (*17*)) KCC3 LOF mutation or knockout disrupts PNS integrity *in vivo* in both humans and mice (*9, 10, 15*); and (vi) the mouse model of the Thr991Ala mutation exhibits motor nerve conduction and locomotor deficits, as demonstrated by the accelerated rotarod and beam walk neurobehavioral tests. The fact that no significant quantitative difference could be observed on either the wire hang nor the force grip tests suggest that the deficit is predominantly affecting the mouse hindlimbs. The observation that homozygous mice required their 4 limbs with or without the tail to remain hanging on the

wire indicates however some forelimb weakness. In contrast to the human patient, we only observed locomotor deficits in the homozygous state in mice. Multiple precedents exist for dominant mutations causing human diseases, which can be only recapitulated in the homozygote mouse. Such an example is lamin A (N195K), which in humans causes dilated cardiomyopathy with conduction system disease (*30*) in the heterozygous condition, but requires homozygous loss in mice (*31*).

Importantly, our patient's clinical syndrome is distinct from ACCPN, which features severe brain pathology and both sensory and motor peripheral neuropathies (nearly all patients lack electrophysiological sensory responses and only mildly reduced and slowed motor responses); ACCPN is due to recessive LOF KCC3 mutations commonly found in French Canadians (*7, 10, 11, 32*). In contrast, our patient has an early onset and severe predominantly motor peripheral neuropathy, lacks clinical or radiographic brain pathology, and harbors a *de novo* GOF mutation in KCC3. While the neurological examination of the patient indicated the neuropathy to be predominantly affecting motor function, this does not exclude some deficit in sensory fibers. It is worth pointing that we also detected a trend towards decreased sensory function in the nerve of mice homozygous for the Thr991Ala mutation. Interestingly, ACCPN patients and KCC3 KO mice exhibit axonal swelling of spinal nerve roots and cranial nerves (*7*). This finding, along with the knowledge that Thr991 is dephosphorylated in response to cell swelling to trigger regulatory volume decrease, suggests that either excessive (for example, Thr991Ala GOF mutation) or insufficient KCC3 activity (for example, the LOF mutation that causes ACCPN) – dysregulated KCC3 activity – results in peripheral nerve dysfunction. We speculate this is due to cell volume dysregulation and subsequent neurodegeneration (**Figure 6**). Further analyses of nerve fiber diameters in the Thr991Ala homozygous mice will be needed to assess whether the axons are shrunken upon gain of KCC3 function.

We show in human cells and in mice that this mutation (Thr991Ala) abolishes the WNK1 kinase-dependent inhibitory phosphorylation at this site, mimicking the dephosphorylated species of the transporter induced during pathological cell swelling. Dysregulation and constutive transporter activation likely contributes to a failure of cell volume homeostasis in peripheral nerves and secondary axonal degeneration. We propose this molecular mechanism

defines a novel form of CMT type 2. Phosphorylation-dependent regulation of KCC3 could also impact intracellular Cl^- concentration and thus contribute to neuronal excitability, although in central neurons, this role is mostly accomplished by the related KCC2 cotransporter (*19-23*).

PNS neurons appear to be particularly dependent on volume-regulated KCC3 activity. Possible reasons for this could include the absence of KCC2 (only present in CNS neurons), a relatively higher resting concentration of intracellular Cl^- in PNS neurons, and the presence of functioning aquaporin water channels in PNS, but not CNS, neurons (*25*). Recognition of KCC3-mediated dysregulation as a disease mechanism identifies the transporter as a potential therapeutic target. Hyperactive KCC3 is sensitive to the loop diuretic furosemide, as demonstrated in our patient's fibroblasts. It will be of great importance to identify additional patients with activating KCC3 mutations, as well as to investigate the role of kinase-mediated KCC3 regulation in other forms of inherited or acquired neuropathies. These findings may have relevance for diabetic peripheral neuropathy, associated with accumulation of the intracellular organic osmolyte sorbitol (*33*), which is an activator of WNK1 activity (*34*).

Lastly, WNK1-dependent phosphorylation of KCC2 at Thr906, a residue homologous to KCC3 Thr991 (**Figure 2C**), maintains the depolarizing action of GABA, critical for brain development, by inhibiting KCC2 in immature rat neurons (*35*). Regulated phosphorylation of this related site in KCC2 might be important for human CNS function, similar to our finding here for KCC3 in the PNS.

MATERIALS AND METHODS

Patient recruitment

This study was approved by the Institutional Review Board of the National Institute of Neurological Disorders and Stroke (NINDS) and National Institutes of Health (NIH). Written informed consent was obtained by a qualified investigator. DNA from blood and skin fibroblasts was obtained based on standard procedures. Medical history was obtained and clinical evaluations were performed as part of the standard neurologic evaluation.

Cell culture, Transfections and Cell Treatments

HEK293 (human embryonic kidney 293) and human fibroblast cells were cultured on 10-cm-diameter dishes in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. For transfection experiments, each dish of adherent HEK293 cells was transfected with 20 μL of 1 mg/ml polyethylenimine (Polysciences) (*36*). 36 hours post-transfection cells were stimulated with either control isotonic or hypotonic medium for a period of 30 minutes. Cells were lysed in 0.3 ml of ice-cold lysis buffer/dish, lysates were clarified by centrifugation at 4°C for 15 minutes at 26,000 g and the supernatants were frozen in aliquots in liquid nitrogen and stored at –20°C. Protein concentrations were determined using the Bradford method. Where indicated cells were treated with the indicated concentrations of the SPAK/OSR1 CCT domain inhibitor termed STOCK1S-50699 (*37*), which was purchased from InterBioScreen Ltd.

Antibodies

KCC3 phospho-Thr^{991} [residues 975-989 of human KCC3 phosphorylated at Thr^{991}, SAYTYER(T)LMMEQRSRR]; KCC3 phospho-Thr$^{1039/1048}$ [residues 1032-1046 or 1041-1055 of human KCC3 phosphorylated at Thr$^{1039/1048}$, CYQEKVHM(T)WTKDKYM]. NKCC1 total antibody [residues 1-288 of human NKCC1]; NKCC1 phospho-Thr^{203}/Thr^{207}/Thr^{212} antibody [residues 198-217 of human NKCC1 phosphorylated at Thr^{203}, Thr^{207} and Thr^{212}, HYYYD(T)HTN(T)YYLR(T)FGHNT]; SPAK-total antibody [full-length GST-tagged human SPAK protein]; SPAK/OSR1 (S-motif) phospho-Ser^{373}/Ser^{325} antibody [367-379 of human SPAK, RRVPGS(S)HLHKT, which is highly similar to residues 319-331 of human OSR1 in

which the sequence is RRVPGS(S)GRLHKT]; OSR1 total antibody [residues 389-408 of human OSR1]; ERK1 total antibody [full-length human ERK1 protein]. All antibodies were raised in sheep and affinity-purified on the appropriate antigen by the Division of Signal Transduction Therapy Unit at the University of Dundee, and previously characterized (*17*). Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Pierce. IgG used in control immunoprecipitation experiments was affinity-purified from pre-immune serum using Protein G-Sepharose.

Buffers and Solutions

Buffer A contained 50 mM Tris/HCl, pH7.5 and 0.1 mM EGTA. Lysis buffer was 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) NP-40, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol and protease inhibitors (1 tablet per 50 ml). TBS-Tween buffer (TTBS) was Tris/HCl, pH 7.5, 0.15 M NaCl and 0.2% (v/v) Tween-20. SDS sample buffer was 1X-NuPAGE LDS sample buffer (Invitrogen), containing 1% (v/v) 2-mercaptoethanol. Isotonic high potassium buffer was 95 mM NaCl, 50 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 1 mM Na_2SO_4 and 20 mM HEPES (pH 7.4). Hypotonic high potassium buffer was 80 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1mM Na_2HPO_4, 1 mM Na_2SO_4 and 20 mM HEPES (pH 7.4). Isotonic buffer was 135 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 0.5mM $Na_2SO_4$and 15 mM HEPES (pH 7.5). Hypotonic low chloride buffer was 67.5 mM sodium-gluconate, 2.5 mM potassium-gluconate, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 0.5 mM Na_2SO_4 and 7.5 mM HEPES (pH 7.5).

Phospho-Antibody Immunoprecipitations

KCC3 phosphorylated at the Thr^{991} and Thr^{1048} residue were immunoprecipitated from human fibroblast cell lysates. The phospho-antibody was coupled with protein-G–Sepharose at a ratio of 1 mg of antibody per 1 mL of beads. A total of 2 mg of clarified cellular lysates were incubated with 15 µg of antibody conjugated to 15 µL of protein-G–Sepharose in the presence of 20 µg/mL of lysate of the corresponding dephosphopeptide. Incubation was for 2 hours at 4°C with gentle agitation, and the immunoprecipitates were washed three times with 1 mL of lysis buffer

containing 0.15 M NaCl and twice with 1 mL of buffer A. Bound proteins were eluted with 1X LDS sample buffer.

Immunoblotting

Cell lysates (15 µg) in SDS sample buffer were subjected to electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated for 30 min with TTBS containing 5% (w/v) skimmed milk. The membranes were then immunoblotted in 5% (w/v) skimmed milk in TTBS with the indicated primary antibodies overnight at 4°C. Sheep antibodies were used at a concentration of 1-2 µg/ml. The incubation with phosphospecific sheep antibodies was performed with the addition of 10 µg/mL of the dephosphopeptide antigen used to raise the antibody. The blots were then washed six times with TTBS and incubated for 1 hour at room temperature with secondary HRP-conjugated antibodies diluted 5000-fold in 5% (w/v) skimmed milk in TTBS. After repeating the washing steps, the signal was detected with the enhanced chemiluminescence reagent. Immunoblots were developed using a film automatic processor (SRX-101; Konica Minolta Medical) and films were scanned with a 600-dpi resolution on a scanner (PowerLook 1000; UMAX). Figures were generated using Photoshop/Illustrator (Adobe). The relative intensities of immunoblot bands were determined by densitometry with ImageJ software.

^{86}Rb Uptake Assay in Human Fibroblast Cells and HEK293 Cells

Human fibroblast cells were plated in 12-well plates (2.4 cm diameter/well) and the ^{86}Rb uptake assay was performed on cells that were 80 % confluent. HEK-293 cells were plated at a confluence of 50–60% in 12-well plates (2.4-cm-diameter per/well) and transfected with wild-type full-length KCC3 or a human disease mutant form of full-length flag-tagged KCC3 (Thr991 to Ala). Each well of HEK-293 cells was transfected with 2.5 µl of 1 mg/ml polyethylenimine and 1 µg of plasmid DNA. The ^{86}Rb-uptake assay was performed on the cells at 36 hours post-transfection. In both cases, culture medium was removed from the wells and replaced with either isotonic or hypotonic medium for 15 min at 37°C. Cell medium was removed by means of aspiration with a vacuum pump and replaced with stimulating medium plus inhibitors including 1 mM ouabain and 0.1 mM bumetanide, to prevent ^{86}Rb uptake via the NKCC1 cotransporter, for a further 15 min. After this period, the medium was removed and replaced with isotonic

medium plus inhibitors containing 2 µCi/ml ^{86}Rb, for 10 min, at 37°C. After this incubation period, cells were rapidly washed three times with the respective ice-cold non-radioactive medium. The cells were lysed in 300 µl of ice-cold lysis buffer and $^{86}Rb^{+}$ uptake tracer activity was quantified on a PerkinElmer liquid scintillation analyser.

CRISPR/cas9 generation of KCC3-Thr991Ala mice

A 20 bp sequence (ATATGAGCGCACCC TGATGA, boxed in Figure 4A) located in exon 22 of mouse *Slc12a6* and followed by TGG as proto-spacer adjacent motif was selected for guide RNA targeting sequence. This sequence flanked by *Bbs*I sites was added to a guide RNA sequence in pX330, a vector expressing the guide RNA under a strong U6 promoter and cas9 under a hybrid chicken beta-actin (Cbh) promoter. The vector was injected alongside a 179 bp repair oligonucleotide into 498 mouse embryos. The repair oligo contained 83 bp homology arms, a codon substituting Thr991 to Ala, a unique *Sac*I restriction site and a few additional third base mutations to prevent targeting of cas9 to the repair DNA. Out of 498 embryos injected, 283 were transferred to 13 pseudo-pregnant females thereby generating 54 pups. At weaning, genotyping was done by amplifying a 479 bp fragment followed by *Sac*I digest. Eight *Sac*I-sensitive (positive) animals out of 54 (15%) were identified. We sequenced the *Sac*I containing mutant alleles and identified 4 mice carrying the proper mutation, whereas the other 4 mice carried additional insertions or deletions (see Figure 4C). We selected two lines (#31 and #51) and crossed them to C57BL/6J mice to demonstrate germline transmission. The lines were then further bred to C57BL/6J to dilute any possible off-target effects.

Mouse Fibroblasts

The tip of a mouse tail (5 mm) was minced using sterile fine forceps and sterile razor blades in a 35-mm culture dish containing 2 ml DMEM/F12 containing 350 U/ml penicillin, 350 ug/ml streptomycin, and 2 mg/ml collagenase D (from *Clostridium histolyticum*, Roche Diagnostics, Indianapolis, IN). The 2 ml medium containing small tail chunks was then added to 3 ml identical medium in a 15-ml conical tube and rotated overnight at room temperature. The next day, 5 ml complete DMEM/F12 medium (10% fetal bovine serum, 150 U/ml penicillin, and 150 ug/ml streptomycin) was added to each tube and the remaining large pieces of tails were allowed to sediment by gravity to the bottom of the tube (1-2 min). The supernatant (10 ml) was

recovered, placed in new tubes, and spun at 900 rpm for 4 min. The supernatants were aspirated, the pellet were resuspended in 0.5 ml complete medium, and placed in 24-well plate for growth. Upon confluence, the cells were detached and successively moved to 35-mm and 100-mm plates.

^{86}Rb uptake in mouse fibroblasts

35-mm dishes were coated with 1 ml water containing 0.1 mg/ml poly-D-lysine, overnight in 37^{o}C incubator. The dishes were then rinsed twice with 1 ml water and covered with 2 ml fibroblasts (1 x 10 cm dish resuspended in 16 ml medium or 1:8 splitting ratio) for 2 hour incubation at 37^{o}C to allow the cells to stick. All dishes were plated from the same cell resuspension, thereby guarantying equal seeding. After 2 hours, the dishes were moved to room temperature and preincubated for 15-20 min in a isosmotic solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM HEPES, pH 7.4, 1 mM glucose. At the end of the pre-incubation period, the medium was aspirated and replaced with identical solution containing 100 uM ouabain, 20 uM bumetanide, 1 mCi/ml ^{86}Rb in the presence or absence of 2 mM furosemide. After 15 min ^{86}Rb uptake, the dishes were washed 3 times with ice-cold buffer, exposed to 500 ul 0.25N NaOH for 1 hour, and neutralize with 250 ul acetic acid glacial. Samples of 300 ul were added to 5 ml scintillation fluid in glass vials for ^{86}Rb counts and of 30 ul were used for protein assay (Biorad). Each condition was done in triplicates and K^+ flux was calculated from ^{86}Rb counts and expressed in mmoles K^+ per mg protein per min.

Cell volume experiments

Cell volume changes in patient and parental cells was determined using calcein as a marker of intracellular water volume, as described previously (Adragna et al., 2015b). Briefly, cells on coverslips were incubated with 0.5 μM calcein-AM for 30 min at 37 °C. The cells were placed in a heated (37 °C) imaging chamber (Warner Instruments, Hamden, CT) on a Nikon Ti Eclipse inverted epifluorescence microscope equipped with perfect focus, a 40X Super Fluor oil immersion objective lens, and a Princeton Instruments MicroMax CCD camera. Calcein fluorescence was monitored using a FITC filter set (excitation 480 nm, emission 535 nm, Chroma Technology, Rockingham, VT). Images were collected every 60 sec with MetaFluor image-acquisition software (Molecular Devices, Sunnyvale, CA) and regions of interest (~10-15 cells) were selected. Baseline drift resulting from photobleaching and dye leakage was corrected

as described before (Lenart et al., 2004). The fluorescence change was plotted as a function of the reciprocal of the relative osmotic pressure and the resulting calibration curve applied to all subsequent experiments as described before (Lenart et al., 2004). The HEPES-buffered isotonic solution contained (in mM, pH 7.4): 100 NaCl, 5.4 KCl, 1.3 CaCl2, 0.8 MgSO4, 20 HEPES, 5.5 glucose, 0.4 NaHC03, and 70 sucrose with 310 mOsm determined using an osmometer (Advanced Instruments, Norwood, MA). Anisosmotic solutions (150, 280 mOsm) were prepared by removal or addition of sucrose to the above solution.

Accelerated Rotarod Assay

A neuromotor coordination task was performed using an accelerating rotating cylinder (model 47600: Ugo Basile, S.R. Biological Research Apparatus, Comerio, Italy) in t wo cohorts of mice: 12 wild-type mice and 12 heterozygous Thr991Ala mice, and 8 wild-type, 7 heterozygotes and 7 homozygote mice. The cylinder was 3 cm in diameter and was covered with scored plastic. Mice were confined to a 4 cm long section of the cylinder by gray Plexiglas dividers. Two-Five mice were placed on the cylinder at once. The rotation rate of the cylinder increased over a 4 min period from 4 to 40 rpm. The latency of each mouse to fall off the rotating cylinder was automatically recorded by the device. Mice that remained on the rotarod during the 300 sec trial period were removed and given a score of 300 sec. The test was performed three trials daily for 3 consecutive days, with an intertrial interval of at least 30 min.

Wire hang grip test

To assess forelimb strength and coordination we utilized the wire hang grip test. The wire was 50 cm long and 2 mm in diameter. The string was approximately x cm above the table surface with bedding placed underneath to prevent injury to the mice falling. We trained the mice for 2 trials followed by 3 test trials in one day. The mice were allowed to hang on the wire for a maximum of 60 seconds, but timing ceased when the mouse fell. The mice were scored on latency to fall.

Force grip test

We utilized the Chatillon digital force meter (San Diego Instruments, San Diego, CA) to assess force grip strength in all limbs of the mice. Training and testing were done on the same day as we trained the mice for three practice trials with ten-minute relief periods between each trial,

followed by three test trials. The mice were allowed to initially grip the wire with all four paws and were then pulled horizontally with consistent force to determine grip strength. We normalized grip strength values to account for any variances due to body weight versus grip strength through the formula: $Force_{mouse}$/$body\ weight_{mouse}$.

Balance beam

To assess motor coordination and balance, we utilized a 1-meter long, steel balance beam. We utilized two beams of varying thickness: a standard-sized beam (12 mm), and a thinner beam (6mm), both of which had square cross sections to assess finer motor movements. The beam was placed approximately 50 cm from the ground and positioned between two pillars. At the start of the balance beam, mice began on an open, square platform and ended in an enclosed, black box with bedding as motivation for the mice to cross. We trained the mice for two days (three trials per day for each beam) beginning with the thicker beam (12 mm) and progressing to the thinner beam (6 mm). The mice were tested consecutively on each beam with ten-minute relief periods between each trial. The third day was used as a test day with three trials for each beam. The mice had approximately 60 seconds to traverse the beam and were scored on the neurological scoring system for beam walking adapted from Feeny and Colleagues.(*38*) This scoring system is based on the ability of the mouse to cross the beam accouting for the number of paw slips. The mice received a score ranging from 1-7 based on their ability to complete the task, to place affected limbs on beam, and on the number of paw slips. This neurological scoring system considers, a high score of 7 to be indicative of a wild type mouse phenotype with no coordination deficits, and a low score of 1 indicative motor defects (score of 7: mouse crosses beam with no more than two paw slips; 6: is able to cross beam but uses affected limbs more than halfway along beam; 5: is able to cross beam but uses affected limbs less than halfway along beam; 4: crosses beam and is able to place affected limbs at least once on horizontal beam; 3: crosses beam but drags (affected) hind limbs; 2: is unable to cross beam, but is able to hold horizontal balance for at least 5 seconds; 1: is unable to cross beam).

Nerve conduction studies

We measured the nerve conduction amplitude and latency in both sensory and motor neurons of the mouse tail and the sciatic nerve. Mice were anesthetized with isoflurane vaporizer (Vaporizer

Sales & Service Inc., Rockmart, GA) with a continual flow of oxygen. Body temperature was maintained with a warming pad. For tail nerve conduction studies, we determined tail motor latency through proximal stimulation using single Nicolet 0.4 mm steel needle electrodes (Rythmlink International LLC, Columbia, SC) with an additional grounding electrode. For sensory and motor sciatic nerve measurements, we stimulated proximally at the sciatic nerve. For all nerve conduction measurements we stimulated with stimulus duration of 20 ms and a range of 25 mA. We assessed latencies and traces using the Viking Software where latency is measured from initial onset to maximum negative peak.

Transmission Electron Microscopy

Sciatic nerve were dissected from adult mice and post-fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate for one hour at room temperature (RT), and then at 4°C overnight. The Vanderbilt EM core further processed the sciatic nerves samples by washing them and post-fixing in 1% osmium tetroxide solution for 1h at RT, and then with .5% osmium for 24 hours. The tissue samples underwent a series of ethanol dehydration steps (50% 5 min, 75% 15 min, 95% 2×15 min, 100% 3×20 min) before they were embedded in Spurr resin at 60°C for 24-48 h. Semi-thin sections (500 nm) were stained with toluidine blue and examined for positioning. Ultra-thin sections (80 nm) were then stained with uranyl acetate and lead citrate and placed on copper grids. Images were observed using a Phillips/FEI T-12 Transmission Electron Microscope.

Statistic Analysis

Two-way repeated measures ANOVA was used to assess statistical significance among wild type, heterozygous, and homozygous groups of mice for the accelerated rotarod, wire hang grip, force grip, and balance beam behavioral assays, followed by Tukey's multiple comparisons test. For the force grip assay, we divided each Force value obtained by the weight of the mouse ($Force/mass_{mouse}$) to account for any variation due to mass differences. For nerve conduction studies we used one-way ANOVA followed by Tukey's multiple comparisons test to analyze statistical significance among all three genotypes. For all statistical analysis we considered $P < 0.05$ to be statistically significant. GraphPad Prism (version 7.0, GraphPad Software, Inc., La Jolla, CA) was used for all statistical tests.

3D Structure Modeling

The three dimensional structure of human KCC3 C-terminal domain (CTD) was modeled by the I-TASSER server (*39*). Structure templates against the primary sequence KCC3 733-1150 identified from PDB database were used for comparative modeling. The top ranking template is the cytoplasmic domain of a prokaryotic cation chloride cotransporter (PDB accession code 3G40), which shows 21.1% sequence identity with KCC3 CTD. Among the top five models predicted by I-TASSER, the one with good topology and secondary structure assignment and a high C-score was chosen. The secondary structure elements of KCC3 were predicted by PredictProtein. PyMOL was used for molecular visualization.

REFERENCES

1. M. Tazir, T. Hamadouche, S. Nouioua, S. Mathis, J. M. Vallat, Hereditary motor and sensory neuropathies or Charcot-Marie-Tooth diseases: an update. *J. Neurol. Sci.* **347**, 14-22 (2014).
2. A. M. Rossor, M. R. Evans, M. M. Reilly, A practical approach to the genetic neuropathies. *Pract. Neurol.* **15**, 187-198 (2015).
3. C. J. Klein, X. Duan, M. E. Shy, Inherited neuropathies: clinical overview and update. *Muscle Nerve* **48**, 604-622 (2013).
4. B. W. van Paassen, A. J. van der Kooi, K. Y. van Spaendonck-Zwarts, C. Verhamme, F. Baas, M. de Visser, PMP22 related neuropathies: Charcot-Marie-Tooth disease type 1A and Hereditary Neuropathy with liability to Pressure Palsies. *Orphanet J. Rare Dis.* **9:38** (2014).
5. K. B. Gagnon, E. Delpire, Physiology of SLC12 transporters: Lessons from inherited human genetic mutations and genetically-engineered mouse knockouts. *Am. J. Physiol. Cell Physiol.* **304**, C693-C714 (2013).
6. M. Pearson, J. Lu, D. B. Mount, E. Delpire, Localization of the K-Cl cotransporter, KCC3, in the central and peripheral nervous systems: expression in choroid plexus, large neurons, and white matter tracts. *Neuroscience* **103**, 483-493 (2001).
7. T. Boettger, M. B. Rust, H. Maier, T. Seidenbecher, M. Schweizer, D. J. Keating, J. Faulhaber, H. Ehmke, C. Pfeffer, O. Scheel, B. Lemcke, J. Horst, R. Leuwer, H. C. Pape, H. Volkl, C. A. Hubner, T. J. Jentsch, Loss of K-Cl co-transporter KCC3 causes deafness, neurodegeneration and reduced seizure threshold. *EMBO J.* **22**, 5422-5434 (2003).
8. M. Shekarabi, A. Salin-Cantegrel, J. Laganiere, R. Gaudet, P. Dion, G. A. Rouleau, Cellular Expression of the K(+)-Cl(-) Cotransporter KCC3 in the Central Nervous System of Mouse. *Brain Res* **1374**, 15-26 (2011).
9. N. Byun, E. Delpire, Axonal and periaxonal swelling precede peripheral neurodegeneration in KCC3 knockout mice. *Neurobiol. Dis.* **28**, 39-51 (2007).
10. H. C. Howard, D. B. Mount, D. Rochefort, N. Byun, N. Dupré, J. Lu, X. Fan, L. Song, J.-B. Rivière, C. Prévost, R. Welch, R. England, F. Q. Zhan, A. Mercado, W. B. Siesser, A. L. George, J. Horst, A. Simonati, M. P. McDonald, J.-P. Bouchard, J. Mathieu, E. Delpire, G. A. Rouleau, Mutations in the K-Cl cotransporter KCC3 cause a severe peripheral neuropathy associated with agenesis of the corpus callosum. *Nat. Genet.* **32**, 384-392 (2002).

11. G. Uyanik, N. Elcioglu, J. Penzien, C. Gross, Y. Yilmaz, A. Olmez, E. Demir, D. Wahl, K. Scheglmann, B. Winner, U. Bogdahn, H. Topaloglu, U. Hehr, J. Winkler, Novel truncating and missense mutations of the KCC3 gene associated with Andermann syndrome. *Neurology* **66**, 1044-1048 (2006).
12. A. Labrisseau, M. Vanasse, P. Brochu, G. Jasmin, The andermann syndrome: agenesis of the corpus callosum associated with mental retardation and progressive sensorimotor neuronopathy. *Can. J. Neurol. Sci.* **11**, 257-261 (1984).
13. D. Deleu, S. A. Bamanikar, D. Muirhead, A. Louon, Familial progressive sensorimotor neuropathy with agenesis of the corpus callosum (Andermann syndrome): a clinical, neuroradiological and histopathological study. *Eur. Neurol.* **37**, 104-109 (1997).
14. J. Ding, E. Delpire, Deletion of KCC3 in parvalbumin neurons leads to locomotor deficit in a conditional mouse model of peripheral neuropathy associated with agenesis of the corpus callosum. *Behav. Brain Res.* **274C**, 128-136 (2014).
15. M. Shekarabi, R. X. Moldrich, S. Rasheed, A. Salin-Cantegrel, J. Laganière, D. Rochefort, P. Hince, K. Huot, R. Gaudet, N. Kurniawan, S. G. Sotocinal, J. Ritchie, P. A. Dion, J. S. Mogil, L. J. Richards, G. A. Rouleau, Loss of neuronal potassium/chloride Cotransporter 3 (KCC3) Is responsible for the degenerative phenotype in a conditional mouse model of hereditary motor and sensory neuropathy associated with agenesis of the corpus callosum. *J. Neurosci.* **32**, 3865-3876 (2012).
16. J. Rinehart, Y. D. Maksimova, J. E. Tanis, K. L. Stone, C. A. Hodson, J. Zhang, M. Risinger, W. Pan, D. Wu, C. M. Colangelo, B. Forbush, C. H. Joiner, E. E. Gulcicek, P. G. Gallagher, R. P. Lifton, Sites of regulated phosphorylation that control K-Cl cotransporter activity. *Cell* **138**, 525-536 (2009).
17. P. de Los Heros, D. R. Alessi, R. Gourlay, D. G. Campbell, M. Deak, T. J. Macartney, K. T. Kahle, J. Zhang, The WNK-regulated SPAK/OSR1 kinases directly phosphorylate and inhibit the K^+-Cl^- co-transporters. *Biochem. J.* **458**, 559-573 (2014).
18. N. C. Adragna, N. B. Ravilla, P. K. Lauf, G. Begum, A. R. Khanna, D. Sun, K. T. Kahle, Regulated phosphorylation of the K-Cl cotransporter KCC3 is a molecular switch of intracellular potassium content and cell volume homeostasis. *Front. Cell. Neurosci.* **9**, 255 (2015).
19. P. Blaesse, M. S. Airaksinen, C. Rivera, K. Kaila, Cation-chloride cotransporters and neuronal function. *Neuron* **61**, 820-838 (2009).
20. K. Kaila, T. J. Price, J. A. Payne, M. Puskarjov, J. Voipio, Cation-chloride cotransporters in neuronal development, plasticity and disease. *Nat. Rev. Neurosci.* **15**, 637-654 (2014).

21. K. T. Kahle, T. Z. Deeb, M. Puskarjov, L. Silayeva, B. Liang, K. Kaila, S. J. Moss, Modulation of neuronal activity by phosphorylation of the K-Cl cotransporter KCC2. *Trends Neurosci* **36**, 726-737 (2013).

22. K. T. Kahle, E. Delpire, Kinase-KCC2 coupling: Cl^- rheostasis, disease susceptibility, therapeutic target. *J Neurophysiology* **115**, 8-18 (2016).

23. K. T. Kahle, A. R. Khanna, J. Duan, K. J. Staley, E. Delpire, A. Poduri, The KCC2 cotransporter and human epilepsy: Getting excited about inhibition. *Neuroscientist* (2016).

24. M. A. Gonzalez, R. F. Lebrigio, D. Van Booven, R. H. Ulloa, E. Powell, F. Speziani, M. Tekin, R. Schüle, S. Züchner, GEnomes Management Application (GEM.app): a new software tool for large-scale collaborative genome analysis. *Hum. Mutat.* **34**, 842-846 (2013).

25. K. T. Kahle, A. R. Khanna, S. L. Alper, N. C. Adragna, P. K. Lauf, D. Sun, E. Delpire, K-Cl cotransporters, cell volume homeostasis, and neurological disease. *Trends Mol. Med.* **21**, 513-523 (2015).

26. J. M. Schwarz, C. Rödelsperger, M. Schuelke, D. Seelow, MutationTaster evaluates disease-causing potential of sequence alterations. *Nat. Methods* **7**, 575-576 (2010).

27. P. C. Ng, S. Henikoff, SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Res.* **31**, 3812-3814 (2003).

28. I. A. Adzhubei, S. Schmidt, L. Peshkin, V. E. Ramensky, A. Gerasimova, P. Bork, A. S. Kondrashov, S. R. Sunyaev, A method and server for predicting damaging missense mutations. *Nat. Methods* **7**, 248-249 (2010).

29. W. Heath, Myelin Sheath Survival following Axonal Degeneration in doubly myelinated nerve fibers. *J. Neurosci.* **11**, 4003-4014 (1991).

30. D. Fatkin, C. MacRae, T. Sasaki, M. R. Wolff, M. Porcu, M. Frenneaux, J. Atherton, H. J. Vidaillet, Jr., S. Spudich, U. De Girolami, J. G. Seidman, C. E. Seidman, Missense mutations in the rod domain of the lamin A/C gene as causes of dilated cardiomyopathy and conduction-system disease. *New Eng. J. Med.* **341**, 1715-1724 (1999).

31. L. C. Mounkes, S. V. Kozlov, J. N. Rottman, C. L. Stewart, Expression of an LMNA-N195K variant of A-type lamins results in cardiac conduction defects and death in mice. *Hum. Mol. Genet.* **14**, 2167-2180 (2005).

32. R. Salin-Cantegrel A, JB, Dupré N, Charron FM, Shekarabi M, Karéméra L, Gaspar C, Horst J, Tekin M, Deda G, Krause A, Lippert MM, Willemsen MA, Jarrar R, Lapointe JY, Rouleau GA., Distal

truncation of KCC3 in non-French Canadian HMSN/ACC families. *Neurology* **69**, 1350-1355 (2007).

33. P. J. Oates, Polyol pathway and diabetic peripheral neuropathy. *Int Rev Neurobiol.* **50**, 325-392 (2002).
34. A. Zagórska, E. Pozo-Guisado, J. Boudeau, A. C. Vitari, F. H. Rafiqi, J. Thastrup, M. Deak, D. G. Campbell, N. A. Morrice, A. R. Prescott, D. R. Alessi, Regulation of activity and localization of the WNK1 protein kinase by hyperosmotic stress. *J. Cell Biol.* **176**, 89-100 (2007).
35. P. Friedel, K. T. Kahle, J. Zhang, N. Hertz, L. I. Pisella, E. Buhler, F. Schaller, J. Duan, A. R. Khanna, P. N. Bishop, K. M. Shokat, I. Medina, WNK1-regulated inhibitory phosphorylation of the KCC2 cotransporter maintains the depolarizing action of GABA in immature neurons. *J. Cell Sci.* **8**, ra65 (2015).
36. Y. Durocher, S. Perret, A. Kamen, High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res.* **30**, e9 (2002).
37. T. Mori, E. Kikuchi, Y. Watanabe, S. Fujii, M. Ishigami-Yuasa, H. Kagechika, E. Sohara, T. Rai, S. Sasaki, S. Uchida, Chemical library screening for WNK signalling inhibitors using fluorescence correlation spectroscopy. *Biochem. J.* **455**, 339-345 (2013).
38. D. M. Feeney, A. Gonzalez, W. A. Law, Amphetamine, haloperidol, and experience interact to affect rate of recovery after motor cortex injury. *Science* **217**, 855-857 (1982).
39. J. Yang, R. Yan, A. Roy, D. Xu, J. Poisson, Y. Zhang, The I-TASSER Suite: protein structure and function prediction. *Nat. Methods* **12**, 7-8 (2015).

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Table 1. Nerve conduction studies. Abnormal results are highlighted in **bold**.

Nerve	Age 7 years			Age 9 years		
Motor Nerve (& muscle)	**Amp (mV); [LLN]**	**CV (m/s); [LLN]**	**DL (ms); [ULN]**	**Amp (mV); [LLN]**	**CV (m/s); [LLN]**	**DL (ms); [ULN]**
Peroneal -EDB	Not done	Not done	Not done	**NR**	**NR**	**NR**
Peroneal -TA	Not done	Not done	Not done	**NR**	**NR**	**NR**
Tibial -AHL	**0.4** [> 2.5]	**NR** [A] [> 40]	**6.1** [< 6]	Not done	Not done	Not done
Median - APB	**1.1** [> 4.5]	**31** [> 50]	**4.8** [< 4.5]	**0.2** [> 4.5]	**14** [> 50]	**5.5** [< 4.5]
Ulnar - ADM	**0.5** [> 4.5]	**14** [> 50]	**4.3** [< 3.5]	**0.1** [> 4.5]	**NR**	**6.3** [< 3.5]
Facial - nasalis	**0.3** [> 1.0]	N/A	**5.8** [<4.2]	Not done	Not done	Not done
Sensory Nerve	**Amp (uV); [LLN]**	**CV (m/s); [LLN]**	--	**Amp (mV); [LLN]**	**CV (m/s); [LLN]**	--
Sural	17 [> 6]	42 [> 40]	--	8 [> 6]	**27** [> 40]	--
Median	**13** [>15]	**44** [> 50]	--	**9** [>15]	**44** [> 50]	--
Ulnar	**7** [> 15]	**33** [> 50]	--	**6** [>15]	**41** [> 50]	--

Abbreviations:
Amp = amplitude
DL = Distal latency
CV = conduction velocity
LLN = lower limit of normal
ULN = upper limit of normal
N/A = not applicable
EDB = Extensor digitorum brevis muscle (peroneal motor innervated muscle in foot)
TA = Tibialis anterior muscle (peroneal motor innervated muscle in leg)
AHL = Abductor hallucis longus muscle (tibial motor innervated muscle in foot)
ADM = Abductor digitorum minimi (ulnar motor innervated muscle in hand)
APB = Abductor pollicis brevis (median motor innervated muscle in hand)
NR = no response
NR^A = no response – no conduction velocity was calculated because the proximal site recording could not be elicited and a velocity could therefore not be calculated

Table 2. Summary of clinical studies of a patient with a KCC3 Thr991Ala mutation.

Test	Result
EMG [1]	Abnormal: Motor > Sensory Axonal Neuropathy
MRI brain [2]	Normal
MR spectroscopy brain	Normal
Serum electrolytes [3]	Normal (except low creatinine)
Urine electrolytes [4]	Normal
Hearing	Normal to speech and pure tones; normal tympanometry and auditory brainstem response
Osmotic fragility	Normal erythrocyte osmotic fragility
Peripheral blood smear	No acanthocytes; normal smear
Creatine kinase	Normal [136]

[1]**EMG** – see Table 1 for specific nerve conduction study data and interpretation.
[2]**MRI brain**– see Figure 1 for picture of normal MRI brain including normal brain volume and corpus callosum.
[3]**Serum electrolytes** - Specific values are as follows: [Na = 138; K = 3.9; Cl 100; HCO_3^- = 23, BUN = 9; Creatinine = **0.12 L** (normal 0.3-0.7)];
[4]**Urine electrolytes -** Specific values are as follows: [Urine Na = 93; Urine K 86.5; Urine Cl = 124]

Table 3. Nerve conduction measurements in mice

Measure		w.t.	hetero	homo	mice	w.t. vs het	w.t. vs homo	Het vs homo
Amplitude	Sensory tail	257 ± 27	227 ± 27	180 ± 11	n = 6	P > 0.05	P > 0.05	P > 0.05
	Sensory nerve	274 ± 56	192 ± 15	226 ± 47	n = 6	P > 0.05	P > 0.05	P > 0.05
	Motor tail	2981 ± 401	3434 ± 447	814 ± 146	n = 6	P > 0.05	< 0.001 ***	< 0.001 ***
	Motor nerve	6381 ± 1296	3573 ± 731	4756 ± 1223	n = 6	P > 0.05	P > 0.05	P > 0.05
Latency	Sensory tail	1.58 ± 0.06	1.61 ± 0.04	1.70 ± 0.09	n = 6	P > 0.05	P > 0.05	P > 0.05
	Sensory nerve	1.88 ± 0.33	1.71 ± 0.36	2.11 ± 0.15	n = 5	P > 0.05	P > 0.05	P > 0.05
	Motor tail	3.20 ± 0.27	3.20 ± 0.53	4.80 ± 0.40	n = 6	P > 0.05	0.0299 *	0.0299 *
	Motor Nerve	2.74 ± 0.37	2.51 ± 0.09	3.50 ± 0.48	n = 6	P > 0.05	P > 0.05	P > 0.05

Sensory and motor signals were measured in dorsal caudal nerve (tail) and sciatic nerve (nerve) in wild-type (w.t.), heterozygous (hetero), and homozygous (homo) mice. One-way ANOVA followed by Tukey's Multiple Comparison Tests.

FIGURE LEGENDS

Figure 1. Brain and muscle imaging of a patient with a KCC3 Thr991Ala mutation. (*A*) T1 sequence brain MRI, mid-sagittal view (*B*) FLAIR sequence brain MRI, axial view, demonstrating normal brain volume. (*C*) Muscle ultrasound (performed on Siemens AcusonS2000) of the tibialis anterior (TA) muscle from a healthy 10-year-old-boy to represent normal echogenicity and bulk. (*D*) Abnormal muscle ultrasound of the TA muscle from our patient at 7-years-old. In C and D, the green line indicates depth of muscle, and the red line indicates the subcutaneous fat layer

Figure 2. Identification of a *de novo* KCC3 Thr991Ala mutation in a patient with an early-onset, progressive, and severe axonal motor neuron neuropathy. (A) DNA chromatograms illustrating the detection of a heterozygous c.2971A>G mutation in exon 22 of *SLC12A6*, encoding a Thr991Ala substitution in KCC3. (**B**) Evolutionary conservation of amino acid Thr991 in KCC3 across the indicated species. Jap, Japanese. (**C**) Conservation of the homologous residues of amino acid Thr991 in KCC3 in other human KCCs. (**D**) Cartoon of the modeled structure of the human KCC3 C-terminal domain (residues 733-1150), based on homology modeling by I-TASSERusing the prokaryotic cation-Cl^- cotransporter (PDB ID: 3G40) as the template. Residues Thr991 and Thr1048 are highlighted in red and orange, respectively.

Figure 3. Thr991Ala decreases KCC3 phosphorylation by the WNK1-SPAK pathway in HEK293 cells and patient fibroblasts. (**A**) Phosphorylation of wild-type KCC3 (WT) or KCC3 Thr991Ala expressed in HEK293 cells. HEK293 cells were transfected with the indicated constructs and exposed to hypotonic low Cl^- conditions for 30 min. Lysates were subjected to immunoblot with antibodies reocognizing the indicated proteins or phosphorylated proteins. ERK1 served as an unaffected loading control. (**B**) Phosphorylation of endogenous KCC3 and KCC3 Thr991Ala in human fibroblasts. Human fibroblast cells derived from the affected patient (heterozygous for KCC3 Thr991Ala) or his unaffected parental controls (WT) were exposed to

hypotonic low Cl^- conditions for 30 min. Lysates were subjected to immunoprecipitation (IP) with antibodies recognizing either KCC3 pThr991 or KCC3 pThr1048, and immunoprecipitates were immunoblotted with KCC3 total antibody. Lysates were also analyzed for the presence of the indicated proteins and phosphorylated proteins. (**C**) Quantification of the results of the Western blots shown in B, statistically significant differences ($p < 0.05$ as assessed by one-way ANOVA from n = 3) are indicated. Data are shown as mean ± SEM. The quantification (ratio calculation) is based on phosphorylated species of KCC3/total KCC3.

Figure 4. Thr991Ala increases KCC3 activity and impacts cell volume regulation in HEK293 cells and patient fibroblasts. (**A**) Transport activity of wild-type KCC3 (WT) and KCC3 Thr991Ala expressed in HEK293 cells. HEK293 cells were transfected, exposed to low Cl^- in isotonic conditions (basic low Cl^-), hypotonic low Cl^- conditions, high K^+ in isotonic conditions (basic high K^+), or hypotonic high K^+ conditions in the presence or absence of STOCK1S-50699 ("IN": WNK-SPAK inhibitor), for an additional 30 min in the presence of 1 mM ouabain (Na^+/K^+-ATPase inhibitor) and 0.1 mM bumetanide (NKCC1 inhibitor), to functionally isolate KCC activity. K^+ influx is presented in pmoles K^+ per mg protein per min and plotted for both isotonic and hypotonic conditions. (**B**) Activity of endogenous wild-type KCC3 (WT1 and WT2) and KCC3 Thr991Ala in human fibroblasts. Cells were exposed to the indicated conditions and then treated in the same conditions with 1 mM of furosemide ("INN": KCC inhibitor) for an additional 30 min in the presence of 1 mM ouabain and 0.1 mM bumetanide. K^+ influx was measured and analyzed as in A. Bars represent mean ± SEM of one large experiment. Statistical significance was determined by two-way ANOVA followed by Bonferroni post hoc tests (*, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$; ns, not significant or $P > 0.05$). Similar results were obtained in three separate experiments. (C) Left panel: Relative change in cell water volume during acute hypotonic stress in KCC3 WT parental control fibroblasts and KCC3 Thr991Ala patient fibroblasts. Both cell types were exposed to isotonic HEPES-MEM (310 mOsm/kg H_2O), followed by hypotonic HEPES-MEM (150 mOsm/kg H_2O) for 20 min, and then isotonic HEPES-MEM for 5 min. Right panel: Summary data of cell volume increase. KCC3 Thr991Ala patient cells exhibited abnormal regulatory volume decrease compared to KCC3 WT cells. Data are mean ± SEM, n=3 coverslips. *, $p = 0.02$ vs. WT.

Figure 5. Genetically-modified KCC3-Thr991Ala mice exhibits locomotor deficits. (A) Portion of exon 22 of *Slc12a6* targeted for cas9 cleavage. A 20 bp (boxed) target sequence, upstream of TGG as Protospacer Adjacent Motif (PAM), was inserted into the guide RNA. (B) Schematic representation of a 179 bp repair fragment containing two 83 bp arms of recombination flanking a mutated 13 bp fragment. The codon change results in substitution of Thr991 into alanine and the introduction of a *Sac*I restriction site. (C) Sample genotyping gel showing a strong band (top) corresponding to full-length PCR fragment. Arrow shows direction of band migration whereas arrowhead highlights the presence of *Sac*I-digested bands. The PCR fragment in sample #52 is completely digested, indicated that the mouse is homozygote for the *Sac*I site. (D) Sequence of mutant alleles from eight *Sac*I positive mice compared to the wild-type and intended mutant allele (shaded in gray). Four mice (52, 49, 42, 27) have additional bp insertions or deletions. The ^ sign represents a 12 bp (TATGAGCGCACA) insertion upstream of the tyrosine codon. Only mice #22, 51, 31, and 12 had the desired mutation. (E) K-Cl cotransport-mediated K^+ flux was measured under isosmotic conditions in fibroblasts isolated from wild-type and KCC3-Thr991Ala heterozygote mice (progeny of line #31). Flux was measured in triplicate under 0.1 mM ouabain, 20 μM bumetanide, and in the presence or absence of 2 mM furosemide. K-Cl cotransport is defined as the flux detected in the absence of furosemide minus the flux detected in the presence of furosemide. (F) Accelerated rotarod data (7-8 mice per group, age P40, 3 trials a day for 3 days) report the time until the animal falls from the rod. KCC3-Thr991Ala wild-type, heterozygous, and homozygous mice were obtained originated from heterozygous breeding (line #31). Repeated measures two-way ANOVA: $F(1, 13) = 0.6290$, $P = 0.4420$ or ns for WT and heterozygotes, and $F(2, 19) = 7.816$, $P = 0.0033$ or significant for homozygotes.

Figure 6. Genetically-modified KCC3-Thr991Ala mice exhibits hindlimbs and nerve conduction deficits. (A) Response of wild-type (WT), heterozygous (Het) and homozygous KCC3-Thr991Ala (Homo) mice to the 6-mm wide beam walk task. Mice were placed on the beam and allowed to cross to a safe platform. A performance score (1-7, see text) was given to each mouse (left axis). The time was also recorded (right axis). (B) Grip strength force was measured in all three genotypes using a bar attached to a force transducer. Data were corrected

for body weight. The values (Y axis) are in Newtons (N) divided by gram of body weight. (D) Sensory (left axis) and motor (right axis) amplitudes of dorsal-caudal tail nerve responses to 20 or 25 mA stimuli in wild-type, hetereozygous, and homozygous KCC3-Thr991Ala mice. (D) similar data for sciatic nerves. Data were analyzed using one way ANOVA followed by Tukey's post-hoc tests. (E-F) Selected traces of motor conduction in dorsal caudal (tail) nerves from of wild-type and homozygous mice The amplitude (in mV) is measured from the onset of the response peak to the top of the response peak. The latency to response is determined from the onset of the stimulus to the onset of the response peak. Tail nerves were stimulated at 25 mA.

Figure 7. Electron micrographs of sciatic nerve fibers isolated from KCC3 wild-type and Thr991Ala mice. Dissected fragments of sciatic nerves were fixed and processed for electron microscopy. (A) Typical view of a transversally cut nerve fascicle (from wild-type mouse) showing a majority of myelinated fibers and a few unmyelinated fibers. (B-C) Higher magnification of wild-type fibers showing packed myelin sheaths. (D-E) Similar views from homozygous KCC3-Thr991Ala nerves. (F-G) Double myelination pathology observed in homozygous nerves. (H-I) Breakage in myelin observed only in KCC3-T991A homozygous mice. All bars are 500 nm.

Figure 8. Finely-tuned KCC3 activity is required for structure and function of the human peripheral nervous system (PNS). KCC3 activity, schematically represented on a scale from none (0) to maximal (max) activity, is contingent on the amount of KCC3 and a balance between the phosphorylated (inhibited) and dephosphorylated (activated) species of KCC3 in the neuronal plasma membrane. Insufficient KCC3 (for example as occurs in ACCPN, OMIM # 218000) due to LOF KCC3 mutations or as seen in KCC3-knockout mice) or excessive, unregulated KCC3 activity (as in the patient described here with a *de novo* GOF KCC3 Thr991Ala mutation that abolishes WNK1 kinase-dependent inhibitory phosphorylation) results in severe and progressive peripheral axonal neuropathy with secondary demyelinating features, likely from impaired cell volume regulation and subsequent neurodegeneration. Normal humans and mice, as well as ACCPN carriers and KCC3 heterozygous knockout mice, fall within a "functional range" that is free of significant pathology.