Peripheral motor neuropathy is associated with defective kinase regulation of the KCC3 cotransporter

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One Sentence Summary:

Excess KCC3 cotransporter activity compromises motor neuron function in humans and mice.

Human motor neuron neuropathy results from defective WNK-dependentregulation of KCC3 cotransporter activity

OR

Human motor neurons require on WNK-dependent regulation of KCC3 cotransporter activity

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ABSTRACT

Using exome sequencing, we identified a naturally occurring heterozygous c.2971A>G (Thr991Ala) mutation in exon 22 of *SLC12A6*, the gene encoding the K^+-Cl^- cotransporter KCC3. The patient with this mutation had early-onset, progressive, and severe peripheral axonal neuropathy. This mutation abolished the inhibitory phosphorylation of KCC3 by the kinase pathway involving WNK, resulting in a hyperactive form of the cotransporter. Dysregulation of KCC3 would affect cell-volume homeostasis and ion balance. We generated a KCC3-T991A mouse mutant using CRISPR/cas9 technology, and this mouse recapitulated the locomotor deficits observed in the patient. Thus, the peripheral nervous system depends on finely-tuned, kinase-regulated KCC3 activity. Furthermore, these results suggest that impaired cell-volume homeostasis contributes to axonal degeneration in humans.

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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 or motor neurons or both. 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 XXX, and recessive loss-of-function (LOF) mutations in *PMP22* cause cause CMT1A and Hereditary Neuropathy with liability of Pressure Palsies (HNPP), respectively (*4*). Whereas CMT1A is characterized by XXX; HNPP is characterized by XXX.

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The *SLC12A* gene family encodes K^+-Cl^- cotransporters (KCCs), which are electroneutral secondary-active transporters that mediate the regulated extrusion of K^+ and Cl^- ions from cells. As such, they play important roles in cell volume homeostasis, epithelial transport, and neuronal excitability (*5*). KCC3 is encoded by *SLC12A6* and is present 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*).

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Autosomal recessive homozygous or compound heterozygous loss-of-function (LOF) mutations in *SLC12A6*, encoding KCC3, cause the autosomal recessive Mendelian disease termed Agenesis of the Corpus Callosum with Peripheral Neuropathy (ACCPN; OMIM #218000) (*7*, *10*, *11*). ACCPN patients and KCC3-knockout mice exhibit severe peripheral nerve degeneration (*9*, *12-15*); however, ACCPN patients also exhibit severe brain phenotypes, including lack of 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.

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Here, we describe a child with a severe and progressive predominantly motor neuron neuropathy, with normal brain structure and function. The patient has a missense mutation (Thr991Ala) in KCC3. This residue is a regulatory site that, when phosphorylated by a WNK1 -dependent

pathway in normal physiological conditions, inhibits the transporter. The Ala substitution renders this site unavailable for phosphorylation and results in constitutive transporter activity. Under normal conditions, reversible phosphorylation and dephosphorylation of this residue in KCC3 contributes to cell-volume homeostasis with cell swelling promoting the dephosphorylated state of KCC3 to activate the transporter and restore cell volume. Phosphorylation-dependent regulation of KCC3 is also predicted to controls intracellular Cl^- concentrations and thus contribute to neuronal excitability.These observations advance our understanding of KCC3 in human physiology, reveal a critical dependence of motor neurons on kinase-regulated KCC3 activity, and suggest that impaired cell-volume homeostasis is a molecular mechanism of human peripheral motor neuron neuropathy.

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 cruising (walking with balance assistance). 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.

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On examination there were no dysmorphic features (including high arched palate, hypertelorism 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.

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Magnetic resonance imaging (MRI) of the brain revealed a normal brain, including an intact corpus callosum, brain stem, cortical folding pattern, and cerebellum (**Figure 1A and 1B**). Comnparison of muscle by ultrasound analysis between the patient and a healthy age- and gender-matched child revealed increased echogenicity (an indication of XXX), 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 various databases of genetic mutations, including dbSNP, NHLBI EVS, ExAC Browser, or GEM.(*16*) Thr991 is conserved among all KCCs and across evolution (**Figure 2B, C**).

Thr991 resides in the KCC3 cytoplasmic C-terminus, a regulatory domain of transporter activity (*17*). 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. XXXX Ala substitution at this site is predicted to have deleterious effects on protein function by MutationTaster, SIFT, and PolyPhen-2.

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Given the importance of Thr991 phosphorylation for swelling-regulated changes in KCC3 activity (*18, 19*), 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-T991A 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 (*19*). Relative to parental fibroblasts, patient fibroblasts showed ~50% less phosphorylation at Thr991 (**Figure 3B, C**), consistent with the heterozygous status of the KCC3 Thr991Ala mutation. XXXXX (Fig. 3A-B).

To confirm that the mutation altered KCC3 function. We assessed KCC3 activity by measuring ^{86}Rb uptake under various conditions. XXXX . Indeed, KCC3 Thr991Ala exhibited significantly higher activity than wild-type KCC3 in all conditions, as assessed by Cl^- and furosemide-sensitive ^{86}Rb uptake studies (**Figure 4A, 4B**). We also assessed the effect of inhibition of the WNK-SPAK pathway, which is responsible for phosphorylation of this residue in KCC3, and the effect of a general KCC inhibitor. XXXXXThr991Ala resulted in significant KCC3 activity even in normally inactivating conditions (**Figure 4A 4B**).

Using CRISPR/cas9, we created a mouse that reproduces the T991A 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-T991A 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-T991A heterozygote siblings, to the accelerated rotarod test to assess motor function. The mice heterozygous for the mutant allele

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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 then tested and again no statistical difference was observed between wild-type and heterozygote mice (repeated measures two-way ANOVA: F(1, 13) = 0.6290, *P* = 0.4420 or ns). However, we observed significant locomotor deficit in the homozygous mice (**Figure 5F**, repeated measures two-way ANOVA: F(2, 19) = 7.816, *P* = 0.0033).

XXXX

DISCUSSION

Phosphorylation of Thr991 and Thr1048 is a key mechanism controlling KCC3 activity (*18, 19*). KCC3 is inactive in isotonic conditions due to WNK1-SPAK -dependent inhibitory phosphorylation at Thr991 and Thr1048 (*18, 19*). Cell swelling causes rapid dephosphorylation of these sites, mediated by protein phosphatase1 (PP1) and protein phosphatase 2A (PP2A) in parallel with increased KCC3 activity to maintain cell volume in vitro (*18, 19*). Ala substitutions at these sites prevent phosphorylation and cause constitutive activity (*18, 19*). Computer modeling revealed a close proximity of Thr991 to Thr1048 in KCC3 (**Figure 2D**). The human mutation Thr991Ala is the *in vivo* equivalent of these *in vitro* experiments and the first such mutation discovered in humans.

Our combined clinical, genetic, 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 mutation is *de novo* and novel (that is it is not a variant represented in the patient's parents or the general population, because it is not seen in over 124,000 *SLC12A6* alleles represented in the ExAC database); (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

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key mechanism that regulates KCC3 activity and cell volume homeostasis (*18*, *19*); (v) Thr991Ala prevents this phosphorylation and results in a GOF effect in patient fibroblasts, paralleling effects *in vitro* (**Fig 3**, (*19*)) and KCC3 LOF mutation or knockout disrupts PNS integrity *in vivo* in humans and mice, respectively (*9*, *10*, *15*); and (vi) the mouse model of the T991A mutation exhibits a significant locomotor deficit. In contrast to the human patient, we only observed locomotor deficits in the homozygous state in mice. Precedents exist for dominant mutations causing human diseases, which can be only recapitulated in the homozygote mouse. Such an example is laminA (N195K), which in humans causes dilated cardiomyopathy with conduction system disease (*20*) in the heterozygous condition, but requires homozygous loss in mice (XX).

Importantly, our patient's clinical syndrome is distinct from ACCPN, which features severe brain pathology and both sensory and motor neuropathy (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*, *21*). In contrast, our patient has a severe early onset progressive motor predominant neuropathy, lacks apparent clinical or radiographic brain pathology, and harbors a *de novo* GOF mutation in KCC3. 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, likely due to a failure of regulatory volume decrease and subsequent neurodegeneration (**Figure 6**). This suggests impaired cell volume homeostasis contributes to both sensory and motor peripheral neuropathy.

Compared to CNS neurons, PNS neurons appear to be particularly vulnerable to disruptions of cell volume and dependent on KCC3. Reasons for this could include the absence of KCC2 and 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 (*17*). These findings may have relevance for diabetic peripheral neuropathy, associated with accumulation of the

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intracellular organic osmolyte sorbitol (*22*), which is an activator of WNK1 activity (*23*). 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 (*24*). 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.

In conclusion, our report presents the first patient with a *de novo* GOF mutation in KCC3, which results in a severe and progressive motor-predominant neuropathy. This mutation (Thr991Ala) abolishes 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. 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.

MATERIAL AND METHODS

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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) (*25*). 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 (*26*), which was purchased from InterBioScreen Ltd.

^{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-T991A 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 odon 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 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.

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 T991A 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.

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Author contributions: XXXX
Competing interests: XXXX
Data and materials availability: XXXX

Commented [NG26]: List anyone providing reagents or other assistance.

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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 [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
NR

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

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Test	Result
EMG [1]	Abnormal: Motor > Sensory Axonal Neuropathy
MRI brain [2]	Normal
MR spectroscopy brain	Normal
Serum electrolytes	Normal (except low creatinine) [Na = 138; K = 3.9; Cl 100; HCO_3^- = 23, BUN = 9; Creatinine = 0.12 L (normal 0.3-0.7)]
Urine electrolytes	Normal [Urine Na = 93; Urine K 86.5; Urine Cl = 124]
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 Supplementary Table 2 for specific nerve conduction study data and interpretation.

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[2]MRI brain– see Figure 1 for picture of normal MRI brain including normal brain volume and corpus callosum.

FIGURE LEGENDS

General comment: Avoid interpretation in the figure legends.

Figure 1. Brain and muscle imaging of a patient with a KCC3 Thr991Ala mutation. (*A*) T1 sequence brain MRI, mid-sagittal view, demonstrating a normal corpus callosum (red arrow), brain stem, cortical folding pattern, and cerebellum. (*B*) FLAIR sequence brain MRI, axial view,

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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 (T991A) 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 a 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< XX as assessed by an unpaired

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t-test fromn=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 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), an inhibitor of WNK-SPAK kinase signaling 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. Statistical significance was determined by ANOVA with post-hoc Student's T tests (*, P < XX; **, P < XX; *** P < XX Data are , ns, not significant). Data are shown as XXX. Similar results were obtained in three separate experiments.

Figure 5. Genetically-modified KCC3-Thr991Ala mouse 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*) Sequence of mutant alleles from eight *Sac*Ipositive 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

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a 12 bp (TATGAGCGCACA) insertion upstream of the tyrosine codon. Mice 22, 51, 31, and 12 had only the desired mutation. (*D*) Genotyping gel showing a strong band (bottom) corresponding to full-length PCR fragment. Arrow shows direction of band migration whereas arrowhead highlights the presence of *Sac*I-digested bands. Tthe PCR fragment in sample #52 is completely digested, indicated that the mouse is homozygote for the *Sac*I site. (*E*) K-Cl cotransport-mediated K^+ flux was measured under isosmotic conditions in fibroblasts isolated from wild-type and KCC3-T991A heterozygote mice (#XX). 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-T991A homozygous mice are from strain #XX, heterozygous mice are from strain #XX. Statistical analysis by two-way ANOVA.

Figure 6. 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.

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