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# KCC3 DEFICIENCY-INDUCED DISRUPTION OF PARANODAL LOOPS AND IMPAIRMENT OF AXONAL EXCITABILITY IN THE PERIPHERAL NERVOUS SYSTEM

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**Abstract—The autosomal recessive Hereditary Motor and Sensory Neuropathy with Agenesis of the Corpus Callosum (HMSN/ACC) is associated with the dysfunction of the $K^+$–$Cl^-$ cotransporter type 3 (KCC3), which is an electroneutral cotransporter. We previously found that the inhibition of KCC3 cotransporter activity reduces the propagation of action potentials in the peripheral nervous system (PNS). However, the pathogenesis by which KCC3 deficiency impairs peripheral nerve function remains to be examined. Thus, we conducted imaging and electrophysiological studies in the peripheral nerves of *KCC3*$^{-/-}$ mice at various ages. Analysis using transmission electron microscopy (TEM) revealed an age-dependent progressive swelling of microvilli and disorganization of paranodal loops in *KCC3*$^{-/-}$ nerves. Yet, no mislocated voltage-dependent channels were observed between the nodes and juxtaparanodes of *KCC3*$^{-/-}$ nerves. However, electrophysiological studies using the threshold tracking technique indicated a reduced stimulus–response curve slope with an elevated rheobase, a decreased strength–duration time constant, diminished persistent $Na^+$ currents, and an outward deviation of threshold electrotonus in *KCC3*$^{-/-}$ nerves compared to wild-type nerves. These functional changes indicate an overall reduction in axonal excitability and suggest an increase in paranodal conductance, which was relevant to the pathology at the paranode. Altogether, our findings highlight the importance of KCC3 in maintaining paranodal integrity and in optimizing the propagation of action potentials along peripheral nerves.** 

**Key words: axon excitability, threshold tracking, impulse conduction, potassium chloride cotransporter, Hereditary Motor and Sensory Neuropathy with Agenesis of the Corpus Callosum, neuropathy.**

## INTRODUCTION

Hereditary Motor and Sensory Neuropathy with Agenesis of the Corpus Callosum (HMSN/ACC), also called Andermann syndrome, is an autosomal recessive disease that was first described in 1972. It has several clinical characteristics, including progressive sensorimotor neuropathy, dysmorphic features, mental deficiency, and complete or partial agenesis of the corpus callosum (Mathieu et al., 1990).

HMSN/ACC is associated with a genetic lesion on the solute carrier family 12 member 6 (*SLC12A6*), which encodes for the $K^+$–$Cl^-$ cotransporter 3 (KCC3). This protein mediates the coupled electroneutral movements of $K^+$ and $Cl^-$ across the plasma membrane with concurrent water flux and leads to regulatory volume decreases (Hiki et al., 1999; Mount et al., 1999; Race et al., 1999; Jennings and Adame, 2001; Delpire and Mount, 2002; Howard et al., 2002; Boettger et al., 2003; Adragna et al., 2004; Rust et al., 2007). The homozygous gene knockout of KCC3 can result in a progressive peripheral neuropathy with abnormal nerve conduction and a subsequent development of comparable phenotypes of HMSN/ACC (Howard et al., 2002; Dupré et al., 2003; Byun and Delpire, 2007; Sun et al., 2010). The pathological outcomes, electrophysiological properties, and neurological phenotypes of *KCC3*$^{-/-}$ mice are nearly identical to those observed in patients with HMSN/ACC (Howard et al., 2002).

In a previous study, we demonstrated that the amplitudes of the compound motor action potentials (CMAPs) and nerve conduction velocity were reduced

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*Abbreviations:* ACC, Agenesis of the Corpus Callosum; CMAPs, compound motor action potentials; HMSN, Hereditary Motor and Sensory Neuropathy; KCC3, $K^+$–$Cl^-$ cotransporter type 3; PBS, phosphate-buffered saline; PNJ, paranodal junction; PNS, peripheral nervous system; SDTC, strength–duration time constant; TEM, transmission electron microscopy.

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after KCC inhibition by either a gene knockout approach or by treatment with a KCC inhibitor. Our previous results indicate that KCC3 is involved in optimizing peripheral nerve conduction, which was also supported by the finding that KCC3 is expressed at or around the peripheral axonal node (Sun et al., 2010). Nevertheless, conventional nerve conduction studies provide little information regarding KCC3 deficiency-associated pathophysiology in the peripheral nervous system (PNS).

In this study, we aim to investigate KCC3 deficiency-associated pathology in the sciatic nerve. We also study the involvement of KCC3 in tuning the function of the node and the paranode, which are the most critical elements for the propagation of action potentials along nerves. Using transmission electron microscopy (TEM), we provide compelling evidence indicating the disruption of peripheral paranodal loops in *KCC3*$^{-/-}$ mice. Further electrophysiological assessments indicate that KCC3 mediates the dynamics of axonal excitability. Our findings confirm that deficiency in KCC3 results in an aberrant paranodal structure in the sciatic nerves and leads to an overall reduction in axonal excitability.

## EXPERIMENTAL PROCEDURES

### Mouse husbandry

Mice were housed in individually ventilated cages on a 12-h light–dark cycle and were allowed access to food and water *ad libitum*. Animals were handled in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of National Cheng Kung University (Approval No. 100155). *KCC3*$^{-/-}$ mice were generated through homologous recombination and backcrossed onto C57BL6 mice, as previously described (Howard et al., 2002; Sun et al., 2010). Genotyping was performed by polymerase chain reaction using the following primers: normal KCC3 forward – 5′-GAACTTTGTGTTGATTCCTTTGG-3′, normal KCC3 reverse – 5′-TCTCCTAACTCCATCTCCAGGG-3′, mutant KCC3 forward – 5′-GAACTTTGTGTT GATTCCTTTGG-3′, mutant KCC3 reverse – 5′-TACAA CACACACTCCAACCTCCG-3′. These primer pairs generated 371 kb and 290 kb products, respectively. A total of 45 wild-type mice and 38 knockouts of various ages were used.

### Preparation of sciatic nerves

Mouse sciatic nerves were isolated and prepared as previously described (Sun et al., 2010). In brief, nerves were detached from the spinal cord and muscle and were washed in phosphate-buffered saline (PBS). The epineuria of isolated nerves were peeled under a binocular microscope and air-dried. Dried, teased axons were fixed in 3.7% paraformaldehyde in PBS for 30 min, permeabilized using 0.05% Triton X-100 for 30 min, and then studied using immunofluorescence. Alternatively, nerve tissues that were used for TEM experiments were first fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer for 1 day and then post-fixed in 1% $OsO_4$ in the same buffer for 1 h. After dehydration in ethanol, tissues were embedded in EMbed 812. The 90-nm ultrathin sections were stained using 2% aqueous uranyl acetate in 50% ethanol and lead citrate. Tissue sections were scanned and photographed in a transmission electron microscope (JEM-1400, JEOL Ltd., Tokyo, Japan) at 80 kV.

### Immunofluorescence

Teased nerves were incubated in blocking serum (Invitrogen, Carlsbad, CA, USA) at room temperature for 30 min, followed by incubation with primary antibody overnight at 4 °C. The primary antibodies used in this study were rabbit polyclonal anti-Kv1.2 channel, anti-Nav1.6 channel, anti-Nav1.2 channel (1:100; Alomone Labs, Jerusalem, Israel), goat polyclonal anti-KCC3, anti-Ankyrin G, anti-βIV spectrin, anti-protein 4.1B (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and sheep polyclonal anti-contactin associated protein 2 (CASPR2, 1:100, R&D systems, Minneapolis, MN, USA). The tissues were washed three times in PBS, and then incubated for 1 h at room temperature with Alexa Fluor 488 or 594 fluorescence-conjugated secondary antibodies (1:200; Invitrogen). The slides were mounted and examined under a scanning confocal microscope (FV-1000, Olympus, Tokyo, Japan).

### Electrophysiological study

*KCC3*$^{+/+}$ or *KCC3*$^{-/-}$ mice were anesthetized using pentobarbital (40 mg/kg) in an atmosphere containing 30% oxygen (1–1.5 L/min) and placed on a thermostatically regulated heating pad set to 37 °C. Electrical stimuli were delivered by a constant current stimulator to the sciatic nerve via a round AgCl-coated electrode (area, 0.7 $mm^2$). A 1-cm skin incision on the lateral thigh was made for the insertion of the cathode. The anode was inserted in the ipsilateral flank. The evoked CMAPs were recorded from plantar muscles using needle electrodes implanted into the foot ~0.5 cm apart. A ground electrode was inserted subcutaneously between the stimulating and recording electrodes. Responses were amplified at 0.2–3 kHz (Nicolet Viking IV, Nicolet Biomedical, Madison, WI, USA). CMAP amplitudes were measured baseline-to-peak.

### Measurement of axon excitability

Stimulus–response curves were obtained using test stimuli 1 ms in duration to establish the maximal CMAP. Responses were obtained for every 0.2-mA increment of stimulation current. The slope was determined using the linear equation fitting multiple stimulus–response values between 20% and 80% of maximal CMAP. The $R^2$ value was $\geqslant$0.95. Peripheral axon excitability was assessed using QtracS software (Institute of Neurology, London, UK). The 'threshold' current necessary to evoke a submaximal target potential set to 40% of the maximum CMAP can be automatically tracked using trial-and-error computer feedback.