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A role for KCC3 in maintaining cell volume of peripheral nerve fibers

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Abstract

The potassium chloride cotransporter, KCC3, is an electroneutral cotransporter expressed in the peripheral and central nervous system. KCC3 is responsible for the efflux of K^+ and Cl^- in neurons to help maintain cell volume and intracellular chloride levels. A loss-of-function (LOF) of KCC3 causes Hereditary Motor Sensory Neuropathy with Agenesis of the Corpus Callosum (HMSN/ACC) in a population of individuals in the Charlevoix/Lac-Saint-Jean region of Quebec, Canada. A variety of mouse models have been created to understand the physiological and deleterious effects of a KCC3 LOF. Though this KCC3 LOF in mouse models has recapitulated the peripheral neuropathy phenotype of HMSN/ACC, we still know little about the development of the disease pathophysiology. Interestingly, the most recent KCC3 mouse model that we created recapitulated a peripheral neuropathy-like phenotype originating from a KCC3 gain-of-function (GOF). Despite the past two decades of research in attempting to understand the role of KCC3 in disease, we still do not understand how dysfunction of this cotransporter can lead to the pathophysiology of peripheral neuropathy. This review focuses on the function of KCC3 in neurons and its role in human and health and disease.

Keywords

KCC3; HMSN/ACC; ACCPN; Andermann syndrome; T991A; Peripheral neuropathy

1. Introduction

In the early 1970s, Andermann and colleagues first described an autosomal recessive anterior horn disease associated with agenesis of the corpus callosum (ACC) in a pair of brothers (Andermann et al., 1972). These siblings were brachycephalic and presented with partial syndactyly of the toes, poor muscle bulk, and generalized hypotonia. These brothers also suffered from severe intellectual disability as a result of their ACC. Initially, it was thought that these boys' parents may be closely related, but there was no evidence to support this claim. However, their parents were from Charlevoix and Saguenay Lac-Saint-Jean, regions located outside of Quebec City, known for their high consanguinity rate. Although it

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was unclear which gene was causing the disease or if this was actually the correct diagnosis, it was evident that both parents must be carriers of a mutated allele for both siblings to develop the disease. In 1996, Casaubon and colleagues mapped the disorder to human chromosome 15q13-q15 using a set of 120 microsatellite DNA markers in 14 families (Casaubon et al., 1996; Howard et al., 2002). A few years later, the mapping of *SLC12A6*, the gene encoding the K-Cl cotransporter 3 (KCC3), to human chromosome 15q14 prompted geneticists to sequence the exons of individuals in Andermann syndrome families (Howard et al., 2002). It is now well documented that a genetic mutation in KCC3 (2436delG, Thr813fsX813) causes a loss of function (LOF) resulting in Hereditary Motor Sensory Neuropathy with Agenesis of the Corpus Callosum (HMSN/ACC) in this Canadian population (Howard et al., 2002). Note HMSN/ACC in this population is also referred to as Agenesis of the Corpus Callosum with Peripheral Neuropathy (ACCPN) or sometimes Andermann Syndrome. For this review we will use the term HMSN/ACC. Other non-French Canadian families in other world regions were later identified with additional KCC3 LOF mutations (Uyanik et al., 2006).

KCC3 functions to efflux K^+ and Cl^-, forcing water molecules out of the cell. Although there have been several insights in understanding the presentation and pathology of HMSN/ACC using mouse models (Boettger et al., 2003; Byun and Delpire, 2007) it is still unclear how a disruption in KCC3 ultimately leads to HMSN/ACC. Interestingly, ongoing research on the role of KCC3 in disease has become more complex with the new human case of a gain of function (GOF) mutation in the cotransporter. The individual with the GOF mutation also presents with peripheral neuropathy (PN) but no corpus callosum nor cognitive abnormalities (Kahle et al., 2016). Although both of these diseases are rare, it is important to understand the role of KCC3 in both diseased and non-diseased states to shed light on related neuropathies and on the biology of KCC3. As such, the content of this review will focus on the biology and function of KCC3 as it relates to human health, as well as questioning its postulated role relative to isoforms of KCC3 and its transporter family.

1.1. The cloning of KCC3

When KCC3 was initially cloned, members of the *SLC12A* gene family were conventionally named in order of discovery. However, two unrelated groups were working simultaneously to clone KCC3 and KCC4, and the names and identities of these two cotransporters became confusing. To clarify the literature, we will provide a brief timeline of how these two transporters were discovered.

In 1999, the first paper published by Hiki and colleagues described a novel K-Cl cotransporter from human umbilical vein endothelial cells (Hiki et al., 1999). Since previous reports had already characterized two K-Cl cotransporters: KCC1 and KCC2 (Gillen et al., 1996; Payne et al., 1996) they named this transporter KCC3. Concurrently however, Mount and colleagues, unaware of the upcoming publication by Hiki, were also publishing a paper that reported the cloning of two new K-Cl cotransporters from human and mouse: KCC3 and KCC4 (Mount et al., 1999). Unfortunately, the KCC3 in the Hiki paper was not the same KCC3 of the Mount paper, but was actually KCC4. After the release of the Hiki paper, David Mount added a note in proof where he indicated that what was termed KCC4 in the

paper should be read as KCC3, and what was termed KCC3 should be called KCC4 (Mount et al., 1999) and the sequences were submitted with corrected names to NCBI. Following these two initial releases, a third paper by Race and coworkers also reported the cloning and characterization of KCC3 from a human placenta cDNA library (Race et al., 1999). In contrast to the KCC3 isoform cloned by Mount and Race, which is driven by the most upstream gene promoter and contains exon 1a, Hiki's KCC3 originated from a more proximal promoter and contained exon 1b (Race et al., 1999). KCC3a is mostly expressed in brain, whereas KCC3b is expressed in kidney (Pearson et al., 2001).

1.2. KCC3: its function and expression

KCC3 is a member of the cation-chloride cotransporters (CCC) or *SLC12A* gene family. This is a family of electroneutral cotransporters that regulate the influx and efflux of ions across plasma membranes for *trans*-epithelial ion transport, maintaining intracellular Cl^- ($[Cl^-]_i$), and maintaining or regulating cell volume (Cruz-Rangel et al., 2011). This *SLC12A* gene family consists of Na^+-dependent and Na^+-independent transporters (Fig. 1), all of which contain 12 transmembrane domains separating large intracellular termini. Two *SLC12A* members, CCC8 and CCC9, are proteins with no defined function. CCC9 might consist of 11 transmembrane domains and a possible extracellular C-terminus (Gamba, 2005). The intracellular termini of SLC12A cotransporters contain important sites of phosphorylation involved in transport activation or inactivation. KCC3 has two major phosphorylation sites, T1048 and T991, that are located in the carboxyl-terminus (Rinehart et al., 2009). The major regulators of the cation-chloride cotransporters are the WNK-SPAK/OSR1 kinases (With No lysine (K), SPS1-related Proline/Alanine Kinase, Oxidative Stress-Responsive kinase 1) (De Los Heros et al., 2014; Moriguchi et al., 2005; Thastrup et al., 2012). The WNK kinases (WNK1-4) phosphorylate SPAK/OSR1, which in turn, phosphorylate key regulatory sites in KCC3 and in the N-terminal tail of NKCC1 (Hannemann and Flatman, 2011). These kinases have opposite effects on the Na^+-dependent and Na^+-independent cotransporters to modulate ion transport and regulate cell volume. Specifically, phosphorylation of these active sites inactivates KCC3 and activates NKCC1, whereas dephosphorylation activates KCC3 and inhibits NKCC1 (Fig. 1).

As a part of the Na^+-independent branch, KCC3 functions to transport one K^+ ion with one Cl^- ion out of the cell per transport cycle. This tightly coupled transport is electroneutral, meaning it does not directly affect neuronal membrane potential. K-Cl cotransport also drives the movement of obligatory water molecules. Whether water moves through the cotransporters or through other pathways is still a matter of debate. However, KCC4 is estimated to transport 500 water molecules per $1K^+$ and $1Cl^-$ (per cycle) (MacAulay et al., 2004). Following cell swelling, K-Cl cotransport is activated, leading to the loss of K-Cl and water, thereby allowing the cell to regain its volume. In neurons of the central nervous system (CNS) and peripheral nervous system (PNS), KCC3 is thought to maintain cell volume. The cotransporter might also participate in the regulation of $[Cl]_i$, although KCC2 predominately accomplishes this latter function in central neurons (DeFazio et al., 2000; Pellegrino et al., 2011).

Initial localization of KCC3 was done in the mouse nervous system. KCC3 is expressed in multiple CNS and PNS cell types but at varying time points throughout development and through adulthood (Table 1). In adulthood, KCC3 is widely expressed in the CNS (Pearson et al., 2001), but highest KCC3 expression has been documented in the amygdala and hypothalamus. In the hippocampus, KCC3 was also found localized in interneurons (Shekarabi et al., 2011). These data are consistent with *in situ* hybridization data in the Allen mouse brain atlas, which show intense antisense oligo staining in neuronal layers of cortical, hippocampal and cerebellar structures. In the Allen spinal cord atlas, positive staining is seen in both dorsal and ventral grey matter. As for the adult PNS, KCC3 is expressed in extremely low amounts in the sciatic nerve and dorsal root ganglion (DRG) (Byun and Delpire, 2007); KCC3 is also expressed in parvalbumin positive (PV+) sensory neurons (Ding and Delpire, 2014). Parvalbumin is expressed in approximately 15—30% of sensory neurons (Zachařová and Paleček, 2009). In a first study published in 2001, we failed to detect KCC3 by Western blot analysis in isolated adult mouse DRG and peripheral nerves (Pearson et al., 2001). However, we later detected KCC3 transcript in DRG neurons isolated from P30 and adult rats using RT-PCR (Byun and Delpire, 2007). Interestingly, through RT-PCR and Western blot analysis of microsomal (or membrane) proteins, we detected KCC3 expression in early postnatal development (P2-P14) but not in adult in sciatic nerves.

The relatively broad expression of KCC3 throughout the brain, spinal cord, and periphery indicates that the cotransporter must play a key physiological role in neurons. Because the activity of the cotransporter is silent under isotonic conditions and activated upon cell swelling, we propose that this role is likely related to cell volume homeostasis. As we will see below, this idea is strengthened by what is observed under disease conditions. Whether all neurons or only a subset of neurons expresses the cotransporter and whether cotransporter function is critical during the entire life of a neuron or during a defined developmental period are questions that remain to be answered.

1.3. Mapping of human KCC3

Mount and colleagues established that the *SLC12A6* gene maps to human chromosome 15q14 (Mount et al., 1999) within the 5 cM (5q13-15) interval that contained the gene(s) responsible for HMSN/ACC (Casaubon et al., 1996). As aforementioned, this led to the sequencing of the *SLC12A6* exons from HMSN/ACC patients and the discovery that KCC3 mutations are responsible for HMSN/ACC (Howard et al., 2002). The affected individuals carry a homozygous deletion of a guanine in exon 18. This deletion (2436delG) leads to a frame shift and premature termination of the open reading frame, ultimately resulting in a loss of the KCC3-mediated transport. Interestingly, one individual was reported to be a compound heterozygote, carrying the 2436delG mutation in exon 18 in one allele and an additional mutation in exon 11 (1584_1585delCTinsG) that results in KCC3 LOF and therefore HMSN/ACC (Howard et al., 2002).

1.4. KCC3 in disease

1.4.1. HMSN/ACC—HMSN/ACC is an autosomal recessive disease since both parents must be carriers of the 2436delG (Thr813fsX813) mutated allele. HMSN/ACC, although rare worldwide, exists at a frequency of 1 in 2117 with approximately 1 in 20 individuals

being carriers in the Charlevoix and Saguenay-Lac-Saint-Jean regions of Quebec (Howard et al., 2002). Thus, it is likely that HMSN/ACC occurred as consequence of a founder mutation (Casaubon et al., 1996).

Defining characteristics of HMSN/ACC include a severe, progressive motor and sensory neuropathy with delays in motor milestones, severe intellectual disability, generalized hypotonia, areflexia, and most individuals losing their ability to walk and becoming bedridden (Casaubon et al., 1996; Howard et al., 2002). What is most peculiar about HMSN/ACC is that individuals can range in agenesis severity, i.e. some individuals present with severe agenesis and no corpus callosum, while affected siblings have a partial or normal corpus callosum.

Post-mortem studies have attempted to classify whether HMSN/ACC is mainly an axonal or a demyelinating neuropathy. It is important to note that HMSN/ACC is considered a sensorimotor neuropathy since patients exhibit severe locomotor and sensory deficits to the same degree (Auer et al., 2016). In the most recent post-mortem study examining eight patients with HMSN/ACC, Auer and colleagues discovered axonomas in both the CNS and PNS (Auer et al., 2016). Axonomas are small regenerative clusters of axons. The presence of axonomas usually indicates that the axon itself is degenerating, hence defining HMSN/ACC as an axonopathy as opposed to a demyelinating neuropathy.

Interestingly, these studies have also revealed that on average, the brain mass of those with KCC3 LOF was significantly greater when compared to matched controls (Auer et al., 2016). This is a significant finding as it indicates that the brain as a whole is accumulating fluid or neuronal debris. This observation is consistent with the idea that KCC3 is involved in regulating cell volume. Post-mortem tissue studies have also pointed to the fact that tissue swelling is often a pathological hallmark of KCC3-mediated disease in humans and mice. For instance, post-mortem reports have also noted swelling of cranial nerve fibers three and seven and of dorsal and ventral roots (Larbrisseau et al., 1984; Mathieu et al., 1990). In a global KCC3 knockout (KO) mouse, we observed fluid filled axons in the sciatic nerves (Byun and Delpire, 2007). A study published recently examined the nodes of Ranvier in the KCC3 knockout mouse. Nodes of Ranvier are myelin sheath gaps that are located along a myelinated axon (Sun et al., 2016). Vacuoles and occasional myelin debris were observed by electron microscopy in the paranodal loops and microvilli of KCC3 KO sciatic nerve fibers. The paranodal loops with large vacuoles displayed detachment from the axon at the node with axon/Schwann cell adhesion underneath being damaged. Note that such damage was absent in the axon/Schwann cell adhesion at the juxtaparanode. These structural defects were shown to affect axon excitability (Sun et al., 2016). The origin of this pathology is difficult to assess. One possibility would be that KCC3 plays a role at the node of Ranvier itself and its disruption causes functional and morphological defects. There is evidence that the cotransporter is expressed at the node, outer mesaxon, and perinuclear nodes in Schwann cells but remains absent in Schmidt Lanterman incisures (Sun et al., 2010). Interestingly, KCC4 expression is also found in the Schwann cell body and mesaxons (Karadsheh et al., 2004) but knockout of KCC4 does not result in locomotor deficit (Boettger et al., 2002). Thus, it is possible that the pathology presents itself at the nodes because these structures are

finely organized and the presence of vacuoles and general axonal swelling might be sufficient to disrupt them.

1.4.2. Mouse models of HMSN/ACC—In efforts to understand HMSN/ACC and the biology of KCC3, multiple research groups have utilized mouse models that have recapitulated the HMSN/ACC-like phenotype (Table 2). In 2002, we created the first global knockout of KCC3 by targeting exon 3, a 95 bp exon, which encodes a fragment of the NH_2-terminal tail of the cotransporter (Howard et al., 2002). We were able to confirm early motor and locomotor dysfunction in the homozygous mouse model; heterozygous mice displayed no abnormalities. Gross anatomical analysis of the brain revealed no agenesis of the corpus callosum in either KCC3 heterozygous or homozygous mice (Howard et al., 2002). Following this first report, Boettger and colleagues reported a KCC3 knockout mouse model where they targeted exons 3 and 4. In addition to the locomotor phenotype, they noted that their mice displayed deafness late in their lifespan, hypertension, as well as a reduced threshold for epileptic seizures (Boettger et al., 2003). They also observed that hippocampal pyramidal cells from KCC3 KO mice displayed an inability to restore their basal cell volume after swelling. Their data showing a shift in the chloride reversal potential (although small compared to the shift observed in the KCC2 knockout) and the inability to volume regulate are consistent with the basic transport function of KCC3 (i.e. intracellular chloride and cell volume homeostasis). Hypertension was later measured and confirmed in the first mouse model by using telemetric catheters to continuously measure heart rate and mean arterial pressure in both light and dark phases. KCC3 deficient mice were characterized by a marked hypertension (30 mm Hg above controls) during both the day and night periods (Adragna et al., 2004). Hypertension in the KCC3 knockout mouse is neurogenic in origin (Rust et al., 2006). The main question that persists is how a disruption in KCC3 function, or rather, disruption in maintenance of cell volume and/or intracellular chloride lead to CNS deficits, nerve pathology and peripheral neuropathy.

1.4.3. KCC3 gain of function—A young boy exhibiting a rare form of peripheral neuropathy (PN) was presented to the Neuromuscular and Neurogenetic Disorders of Childhood Section at the National Institutes of Health (NIH). The patient tested negative for all known mutations associated with Charcot Marie Tooth Disease, a well characterized inherited neuropathy. Neither of his parents presented with PN. Additionally, his motor neuropathy appeared greater than his sensory neuropathy, and he displayed no cognitive abnormalities. Whole exome sequencing demonstrated that the boy has a *de novo* heterozygous mutation in KCC3, which leads to the substitution of Thr991 for an alanine (T991A) (Kahle et al., 2016). Extraordinarily, the mutation occurs on one of the two most critical phospho-regulatory residues in KCC3 (Rinehart et al., 2009), resulting in a lack of phosphorylation at T991A and constitutive activity of the cotransporter. Thus, this heterozygous mutation is a gain of function (GOF) mutation. As indicated above, KCC3 activity is silenced upon phosphorylation of T991 and T1048. In the patient, T1048 is intact and still capable of being phosphorylated, yet this is not enough to compensate for the lack of phosphorylation at position 991.

To study this KCC3 GOF and recapitulate the young boy's phenotype, we created a T991A mouse model using CRISPR/Cas 9 technology (Kahle et al., 2016). We conducted locomotor tests with adult mice, comparing wild-type controls with heterozygous and homozygous cohorts. We observed that the homozygous T991A mice displayed severe locomotor deficits as they showed difficulty in maintaining balance on the rotarod and coordination on the balance beam (Kahle et al., 2016). In addition, nerve conduction (compound nerve action potential) experiments were conducted to examine sensory and motor amplitudes and latencies. Homozygous mice displayed a significant decrease in motor amplitude and latency, and displayed a trend in decreasing sensory amplitude. The changes in sensory amplitude did not reach statistical significance (Kahle et al., 2016). The patient displayed a greater motor neuropathy than sensory neuropathy, therefore the T991A mouse model's phenotypes were consistent with the T991A patient. The nerve conduction phenotype observed in both homozygous and heterozygous mice established the deleterious effects of this single point mutation in KCC3 and confirmed causality of the T991A mutation to the patient's neuropathy.

Compound nerve action potential studies are useful in identifying the type of defect associated with a neuropathy (i.e. demyelinating defect versus axonal deficit). Indeed, in axonal neuropathies or neuropathies in which mainly axons degenerate, amplitudes in compound nerve action potential tend to decrease. In contrast, a decrease in latency is typically observed in demyelinating neuropathies, as saltatory conduction is affected (Mallik and Weir, 2005). Thus, we utilized nerve conduction studies as a tool to further understand the T991A mutation. In dorsal caudal tail nerve conduction studies, homozygous mice displayed the lowest motor amplitudes and decreased latencies. These results suggested that both myelin and axons are affected in this GOF mutation. It is noteworthy, however, that axon and myelin degeneration begin to confound one another as they progress, making it difficult to interpret the nerve conduction data. Importantly, individuals with HMSN/ACC (LOF of KCC3) have been characterized as having an axonopathy, based on post-mortem observations (Auer et al., 2016).

Another factor to consider is the physiological relevance of a GOF in KCC3. If KCC3 is continually working to efflux K^+, Cl^-, and water, one would expect the cells to shrink. Thus, just as the loss of KCC3 function results in axonal swelling (Byun and Delpire, 2007), it is likely that GOF of KCC3 leads to shrunken axons. Here, we tested this hypothesis by measuring axon diameters in the sciatic nerves of wild-type, KCC3-T991A heterozygous, and homozygous mice. Sciatic nerves from both genotypes were isolated, fixed, and prepared for electron microscopy (Fig. 2A–B). To quantify nerve fiber diameters, we utilized the g-ratio, which is calculated as the total axon diameter over fiber diameter (Fig. 2C). For g-ratio analysis, nerve fibers are assumed to be perfect circles in order to estimate the diameter. We used Image J software to trace and measure the outer portion of the axon and the outer area of the myelin sheath (Fig. 2D). Interestingly, when plotting axon diameter versus g-ratio, control animals display a greater distributional spread of axon diameters; comparatively, heterozygous and homozygous animals have a majority of their axon diameters concentrated in the smaller axon diameter range (Fig. 3A). Moreover, we observed an overall decrease in myelin thickness in both heterozygous and homozygous mice as well as decreased fiber diameters when compared to control animals (Fig. 3B and

D). Interestingly, this change in distribution points towards cell shrinkage. An overall decrease in axon diameter is consistent with a GOF mutation in KCC3 (Fig. 3D).

1.5. KCC3 as a drug target

If KCC3 function is overactive in the T991A patient, could the neuropathy be treated with drugs that lead to cotransporter inhibition? One possibility would be to use a drug that directly interacts and inhibits the cotransporter (Delpire and Kahle, 2016). Currently, the only FDA approved drugs that inhibit the K-Cl cotransporters are the loop diuretics furosemide and bumetanide. Unfortunately, these drugs are poor inhibitors of the K-Cl cotransporters, with an IC50 for KCC above 500 μM in contrast to IC50s < 2 μM for NKCC1 or NKCC2 (Russell, 2000). A drug that acts as a weak inhibitor, might in fact, be a better strategy. Complete inhibition of KCC3 would mimic a LOF of the cotransporter and could be detrimental to the health of nerves. Whether or not the loop diuretics can reach the CNS or PNS is a question that still needs to be answered. In addition, a pharmacological approach also requires target specificity which is an issue when dealing with four K-Cl cotransporters with different expression patterns and functions. In a large screening effort against KCC2 as a target, we identified inhibitory compounds that were 3–4 orders of magnitude more potent than the two loop diuretics (Delpire et al., 2009). However, these compounds were not ideal drug candidates as they were not specific to KCC2 but also inhibited KCC3, and they had poor pharmacokinetic properties (Delpire et al., 2012, 2009). Alternative to agents that bind to the cotransporter would be agents that affect proteins that modulate cotransporter activity. The most likely targets are the kinases that phosphorylate and inactivate KCC3 (SPAK and OSR1) or their upstream kinases (WNKs). This pathway also constitutes a major challenge as the same kinases which regulate KCC3 in neurons also regulate cation-chloride cotransporters such as KCC2 in neurons (De Los Heros et al., 2014), NKCC1 in neurons (Geng et al., 2009) and other tissues, and NKCC2 and NCC in kidney (Delpire and Gagnon, 2006; Gagnon et al., 2006; Grimm et al., 2012). Therefore, manipulating these kinases would likely create many off-target effects. Finally, whether the KCC3-mediated peripheral neuropathy can be reversed once it begins is also uncertain and should be further investigated before designing drugs to target KCC3 dysfunction.

1.6. The cellular basis of disease: KCC3

1.6.1. Neuronal by nature—The fact that both KCC3 LOF and GOF lead to peripheral nerve disease is indicative that the cotransporter must maintain homeostatic function. Initial studies of HMSN/ACC patients and LOF mouse model studies clearly indicated abnormalities in central and peripheral nervous systems. To further tease apart the cellular basis of HMSN/ACC, Shekarabi and colleagues created a neuronal-specific knockout mouse model of KCC3 using the Cre/LoxP system. Deletion of KCC3 was accomplished by expressing Cre under the promoter for synapsin-1, a neuronal specific protein which is involved in synaptic vesicle release and thus modulation of neurotransmission (Cesca et al., 2010). Thus, by crossing a synapsin-1-Cre mouse with a KCC3-flox mouse, they drove deletion of KCC3 in both CNS and PNS neurons. This neuronal-specific knockout mouse displayed the same phenotypes as the global knockout, presenting the first piece of evidence that the KCC3-mediated neuropathy is neuronal in nature (Shekarabi et al., 2012). To determine if the neuronal-specific deletion had an effect on the corpus callosum, they

utilized gold staining and MRI. When assessing changes in the corpus callosum, the neuronal-specific KCC3 KO mice displayed only slightly smaller corpus callosum lengths and smaller anterior commissure areas, compared to wild-type. In the global KO, the phenotype was enhanced with even smaller corpus callosum lengths, smaller anterior commissure areas, and overall smaller corpus callosum volumes. In spite of the more robust changes in the corpus callosum in the global KCC3 LOF, these brain abnormalities were not as visually apparent as they are in the human HSMN/ACC cases. Therefore, it still remains to be resolved whether a) loss of KCC3 expression in additional cell types participates in this phenotype and b) if KCC3 is directly affecting these changes in the corpus callosum.

Concurrently, we also created a neuronal-specific knockout of KCC3 using the neuronal-specific enolase (NSE or enolase-2) promoter to drive deletion of KCC3 in all neurons (Ding and Delpire, 2014). NSE is one of three glycolytic enzymes expressed in brain metabolism (Rosenstein, 1993) and hence ubiquitously expressed in all neurons. In humans, increased NSE expression correlates with brain injury or with stress, such as seizure episodes (Palmio et al., 2008). Surprisingly, this neuronal-specific KO displayed no locomotor deficits. As enolase-2 and synapsin-1 are expressed in all neurons, there should be a phenotype observed in the enolase-2-Cre x KCC3-flox mouse. There are several possible explanations for this discrepancy: First, the enolase-2 promoter might have been weak, and only a partial deletion of KCC3 might have occurred. Note that heterozygous KCC3 KO mice have no phenotype, indicating that expression has to be reduced at least below 50%. Second, it is possible that the expression of enolase-2 does not occur early enough, and KCC3 plays a critical role during development of the nerve. Indeed, enolase has been found weakly expressed in fetal brain, with expression increasing 1–2 weeks postnatal (Rosenstein, 1993). Furthermore, in one of the original NSE-Cre specific lines, it was found that NSE is restricted to fully differentiated neurons and is detected in DRG past postnatal day 0 (Kwon et al., 2006). Although the authors noted that NSE-driven Cre activity was detectable in the PNS at E13.5, the expression might be too low to affect KCC3 expression in early development. Although KCC3 is expressed in adult DRG, its expression increases with gene maturation (Lucas et al., 2012).

An additional Cre line that our laboratory used to disrupt KCC3 was Nav1.8-Cre. Nav1.8 is a voltage-gated sodium channel that is predominately found in small diameter DRG and has been implicated in nociception, thermoception, and mechanoreception (Liu and Wood, 2011). These are three sensory modalities that are commonly affected in HSMN/ACC. Nav1.8 expression can be detected as early as E15 in dorsal root ganglia, with expression reaching approximately 25% of the DRG population by E17 and increasing up to 50% by adulthood (Benn et al., 2001). Nav1.8 expression, therefore, precedes KCC3 expression during development, and Cre-mediated recombination and excision of KCC3 exon 7 likely occurred early. There was however no significant difference between genotypes in the response time of paw withdrawal following a heat-evoked (52 °C) nociceptive stimulus (Ding and Delpire, 2014). As anticipated for nociceptive fibers, disruption of KCC3 in Nav1.8 positive neurons also had no effect on locomotion.

Aside from the Enolase-2-Cre and Nav1.8-Cre, we also used a Parvalbumin-Cre (PV-Cre) line to mediate Cre recombination and excision of KCC3 exon 7 in all parvalbumin-

containing cells, including parvalbumin-positive neurons. We chose PV to disrupt KCC3 as it is expressed in a subset of sensory neurons: the proprioceptive fibers, which transmit information about body position, motion, equilibrium, and limb posture to the CNS. In addition to these sensory neurons, PV is also expressed in a subset of interneurons (Celio, 1990,1986). PV-positive interneurons have been implicated as gate-keepers that modulate excitatory neurons to alleviate neuropathic pain (Petitjean et al., 2015). We observed severe locomotor deficits, swelling, tissue vacuolization, and fiber degeneration in dorsal root ganglia of PV-Cre x KCC3-floxed mice (Ding and Delpire, 2014).

Interestingly, the two gene promoters that drove Cre expression in the two lines of mice that recapitulated the HSMN/ACC phenotype (the synapsin-1 and parvalbumin promoters) are active as early as E14 in both the CNS and PNS (Melloni and DeGennaro, 1994; Zhang et al., 1990); whereas the enolase promoter seems to be active later, as enolase expression is not detected before P7 (Rosenstein, 1993). These data suggest that the HSMN/ACC phenotype might be due to an early deficit in KCC3 function. Although KCC3 expression has not been studied during embryonic development, we know that the transporter is expressed as early as P2 in DRG (Byun and Delpire, 2007). Additional studies are needed to address the precise timing of KCC3 deficit in HSMN/ACC.

1.6.2. A role for Schwann cells?—To further tease apart cell specificity in the development of HMSN/ACC, we also utilized a Desert hedgehog (Dhh)-Cre mouse to delete KCC3 specifically in Schwann Cells, the cells that form and maintain the myelin sheaths in the PNS. Desert Hedgehog is the protein that signals and initiates Schwann Cell formation around peripheral nerves (Parmantier et al., 1999). While antibody studies have demonstrated a definite expression of KCC4 in Schwann cells and mesaxons along teased nerve fibers (Karadsheh et al., 2004), KCC3 transcript was also detected by RT-PCR in freshly isolated Schwann cells (Byun and Delpire, 2007; Karadsheh et al., 2004). Interestingly, the Schwann cell specific mouse knockout of KCC3 displayed no locomotor deficits nor did it display any tissue abnormalities. At the time of this writing, this is the only KCC3 mouse model in literature that disrupts KCC3 in a glial cell subtype. Since KCC3 is involved in volume regulation, we initially hypothesized that perhaps KCC3 might regulate volume in Schwann cells, but KCC3 does not appear to play a prominent role in the volume homeostasis of Schwann cells. Moreover, no attempts have been made to disrupt KCC3 function in oligodendrocytes, the cells that create the myelin sheath the CNS. Since post-mortem autopsies of HMSN/ACC individuals and of mouse models appear to predominantly show abnormalities in axons (i.e. swelling), and the phenotype can be reproduced by deleting KCC3 in cells expressing synapsin-1 or parvalbumin, it is now clear that HMSN/ACC is primarily neuronal in nature (Byun and Delpire, 2007).

The next question that needs to be addressed is whether the phenotype is strictly related to proprioceptive fibers or if other neuronal cell types contribute to the development of HMSN/ACC. Since patients display severe sensorimotor neuropathy it seems logical that both sensory and motor neurons would play a role in the development of the peripheral neuropathy. It is also important to recall here that HMSN/ACC was first presented as an anterior horn disease (Andermann et al., 1972), questioning whether motor neurons also play

a role. Do motor neurons also express KCC3, and would deletion of KCC3 in these neurons lead to a locomotor phenotype? This is yet to be determined.

1.7. The future of KCC3 and HMSN/ACC

Thus far, literature in the field indicates that KCC3 dysfunction in neurons, either GOF or LOF, leads to a neuropathy phenotype. However, the nervous system is complex and multiple components are often involved in sophisticated physiological processes. The parvalbumin Cre/LoxP work, while a promising lead, must be further developed before concrete conclusions can be drawn among KCC3, parvalbumin-positive neurons, and HMSN/ACC. Perhaps, then, it is prudent to reexamine the disease characteristics to guide future studies.

At first glance, the characteristics of HMSN/ACC seem discordant; some patients have corpus callosum malformation while others do not (Howard et al., 2002; Casaubon et al., 1996), and a patient with an intact corpus callosum can still suffer from intellectual disability. Therefore, the most consistent feature of the disease is the sensorimotor neuropathy, yet the neuropathy itself presents another layer of nuance. Is the phenotype caused by sensory neuron deficits, motor neuron deficits, or a combination of the two?

In the periphery, muscle contraction is accomplished by the action of primary motor neurons, which have their cell bodies in the ventral horn of the spinal cord, onto muscles (Stifani, 2014) (Fig. 4). In contrast, sensory neurons, specifically proprioceptive neurons, bring sensory information from the muscles back to the spinal cord, where they synapse onto both primary motor neurons and interneurons. Thus, sensory feedback influences motor neuron control of muscles at the level of the spinal cord. Proprioceptive feedback also travels to the CNS via the dorsal spinocerebellar and medial lemniscal tracts and eventually makes its way to the motor cortex (Fig. 4). In the motor cortex, proprioceptive information can be used to modulate the control of primary motor neurons, marking a second influence of sensory feedback on motor neurons, this time in the brain. Thus, motor neurons and sensory neurons are both connected to the motor cortex via descending and ascending pathways, respectively (Kandel et al., 2000).

Considering the connectivity between the brain and the spinal cord, how can this information be used to investigate the HMSN/ACC pathology? The most salient clinical feature of HMSN/ACC patients is the severe locomotor deficit. Therefore, it seems logical that KCC3 dysfunction in motor neurons may be the cause. The neuromuscular junction (NMJ) of KCC3 KO mice was recently investigated by Bowerman and colleagues (Bowerman et al., 2017). When examining the tibialis anterior muscle, they found that some 20% of KCC3 global KO NMJs were partially denervated. Both KCC3 global KO and neuronal-specific KCC3 KO mice showed disorganized motor endplates. Additionally, the nerve terminals of KCC3 KO mice were shrunken compared to wild-type mice. However, they did not find a difference in the number of motor neuron cell bodies in the ventral horn of the spinal cord when comparing KCC3 KO to wild-type mice, indicating that the differences seen at the NMJ were not due to motor neuron death. Overall, the work by Bowerman and colleagues shows that KCC3 global KO mice have deficits at the NMJ, and considering the locomotor deficits seen before in this mouse line, it is likely that these NMJ

deficits contribute to the phenotype of the mouse. However, it is still unclear whether these deficits are the primary reason behind the locomotor deficits or whether they are a secondary result of deficits in another area of the brain-spinal cord circuitry.

When Andermann and colleagues first described HMSN/ACC in 1972, they believed the patients may have been suffering from anterior horn cell disease, referring to the motor neurons of the anterior spinal cord. They suggested this diagnosis because patient electromyographs displayed a decrease in the number of motor unit potentials but an increase in amplitude of the unit potentials that were present and some polyphasic potentials. This observation is consistent with the NMJ deficits that Bowerman observed; however, the authors of this latest study did not see any differences in the amplitude or number of action potentials in motor neurons cultured from KCC3 global KO mice.

Yet there are still more locomotor characteristics to be considered in the HMSN/ACC phenotype. Locomotion can be broken down into initiation of motor movements, brief voluntary movements, and rhythmic sustained movements. Brief voluntary motor movements, such as raising an arm, are initiated in the primary motor cortex and pre-motor cortex. The primary motor cortex is involved in planning voluntary movements, and its neurons synapse onto motor neurons in the brainstem (corticobulbar tract) and the skeletal motor neurons in the ventral horn of the spinal cord (corticospinal tract) (Purves et al., 2001). The upper motor neurons of the pre-motor and motor cortexes are unlikely to be involved in the HMSN/ACC phenotype. Typically, upper motor neuron lesions result in muscle spasticity, hyperactive deep tendon reflexes, and abnormal big toe behavior upon sole stimulation or pressure (Babinski's sign) in patients, but do not result in amyotrophy (Purves et al., 2001). Traditionally, a disease that has been considered a purely upper motor neuron disorder is primary lateral sclerosis (PLS), and PLS patients present with hyper-reflexia and increased muscle tone (Wais et al., 2017).

After the action potential for a voluntary movement has left the upper motor neurons and reached the lower motor neurons of the ventral horn, these neurons send signals to skeletal muscles to contract. Patients with lower motor neuron lesions can present with muscle wasting, limb weakness, hypoactive tendon reflexes, and decreased muscle tone (Purves et al., 2001). Additionally, patients with spinal muscular atrophy (SMA), a disease of the alpha motor neurons, display hypotonia in infancy, progressive weakness (especially in the legs), and progressive muscle atrophy (Mercuri et al., 2017). The symptoms of lower motor neuron lesions and SMA are more like the phenotype of HMSN/ACC patients than those of upper motor neuron diseases, suggesting that lower motor neurons may play a role in HMSN/ACC.

Lower motor neurons do not affect voluntary movement in isolation. Other neurons, such as proprioceptive sensory neurons and interneurons also play key roles in body movement, including locomotion. Locomotor activity is a complex behavior that involves rhythmic activation of motor neurons for the flexor and extensor muscles of the limb. Both proprioceptive (type Ia) sensory neurons and interneurons also contribute to the rhythmic activation of flexor and extensor muscles around a joint (Kiehn, 2016). Here, it is important to note that the HMSN/ACC patient phenotype does not offer a clear indication of which pathway is predominantly affected. HMSN/ACC patients experience degrading ambulation

as they age, but it is unknown if they experience this symptom because of a sensory deficit, a motor deficit, or both. Our study with the parvalbumin-specific KCC3 KO mouse suggests a significant role for proprioceptive fibers (Ding and Delpire, 2014). This, however, does not exclude a role for motor neurons as well. Could the motor neuropathy observed in HMSN/ACC patients be secondary to a deficit in proprioceptive fibers? In the absence of proper sensory feedback, the motor neurons might be unable to affect muscle contraction at an appropriate time. In this case, the KCC3 disruption might not directly affect motor neurons, but the end result would be the same, with motor neurons unable to convey proper signals to enable locomotion. Future studies should examine KCC3 dysfunction directly in lower motor neurons and further tease apart the role of KCC3 in proprioceptive fibers.

2. Conclusion

Despite nearly two decades of research and the creation of multiple mouse models, we are still left with many more questions than answers regarding the biology of KCC3 and how its dysfunction can cause HMSN/ACC. This review has clearly demonstrated that both LOF and GOF mutations in KCC3 result in peripheral neuropathy, but it is still mostly unclear how the cotransporter is involved. Moreover, it is still uncertain how ACC may be related to a loss of function of KCC3, but not necessarily a gain of function. It is important to note, however, that complete absence of a corpus callosum is never observed in mouse models. Compared to the various degrees of ACC observed in human patients, the changes observed in the mouse are relatively minor, missed in the analyses of the first two mouse knockout models (Howard et al., 2002; Boettger et al., 2003), but detected upon more rigorous analyses by Shekarabi and colleagues (Shekarabi et al., 2012).

The LOF and GOF mouse models as well as the documented clinical cases all point toward disruption in cell volume. Indeed, nerve fibers are swollen in the KCC3 knockout mouse (and HMSN/ACC patients), while shrunken in the constitutively active KCC3-T991A mouse. When added to our knowledge that the cotransporter is activated by cell swelling and then extrudes water out of the cell, this suggests a role for KCC3 in cell volume maintenance of peripheral nerve fibers. The time point when KCC3 function is critical for neurons or their axons is unknown. Is it needed only during a defined developmental period, or is it required during the entire life of the nerve fibers? Because the transporter is otherwise functionally silent under isotonic conditions, is it possible that KCC3 would be required in developmental processes that lead to cell volume changes, or upon situations like inflammatory or injury conditions that lead to cell swelling? If the transporter is maintaining a key parameter such as cell volume and integrity of a nerve fiber, could it be that its function is also affected in other cases of peripheral nerve disease? Finally, there is the question of disease reversibility and possibility of targeted therapeutic agents. Currently there are no drugs or therapies that specifically target KCC3 function. The challenges that exist with drug targets are twofold. First, there is a list of unknown cell types that could also be affected upon KCC3 disruption. Second, there are other isoforms of the K-Cl cotransporter differentially expressed throughout the body that will also likely be targets of KCC3 therapies. It will be difficult to target KCC3 specifically with the level of conservation existing among isoforms. The challenge with targeting KCC3 will be to

effectively change its function without disrupting or affecting any of its other isoforms or proteins it may interact with.

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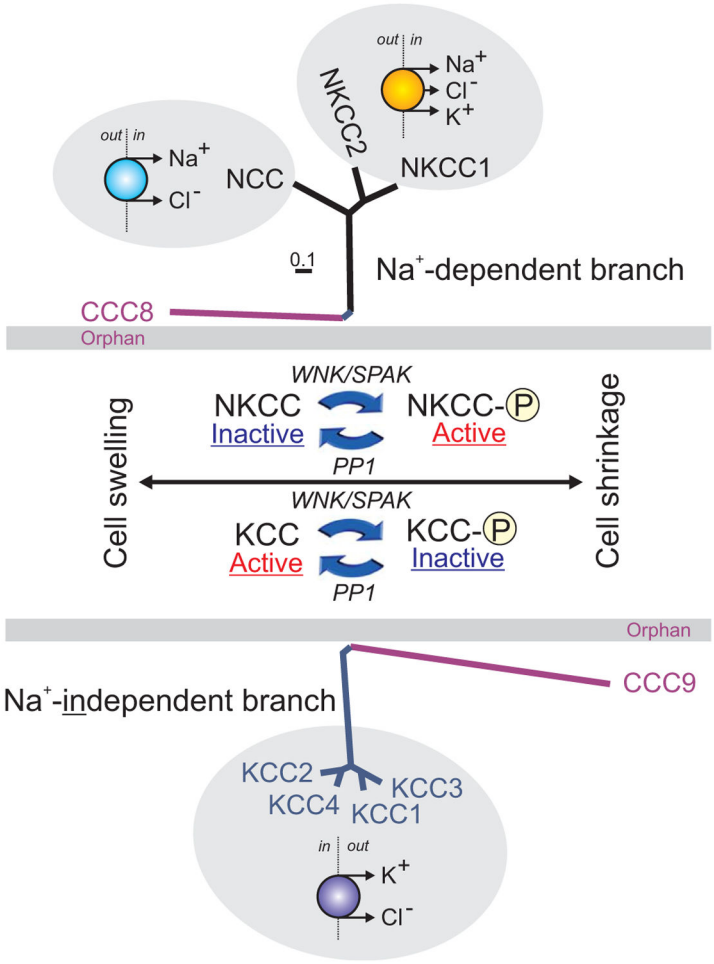

Fig. 1.

The sodium dependent branch works to influx Na^+, Cl^-, and K^+ (NKCC) or Na^+ and Cl^- (NCC) into the cell. The sodium independent branch extrudes K^+ and Cl^- (KCC) from the cell. These cotransporters work in a reciprocal fashion to regulate and maintain intracellular Cl^- and/or cell volume. Phosphorylation by SPAK/OSR1 kinases activates the Na^+-dependent transporters, whereas phosphorylation inactivates the Na^+-independent K-Cl cotransporters.

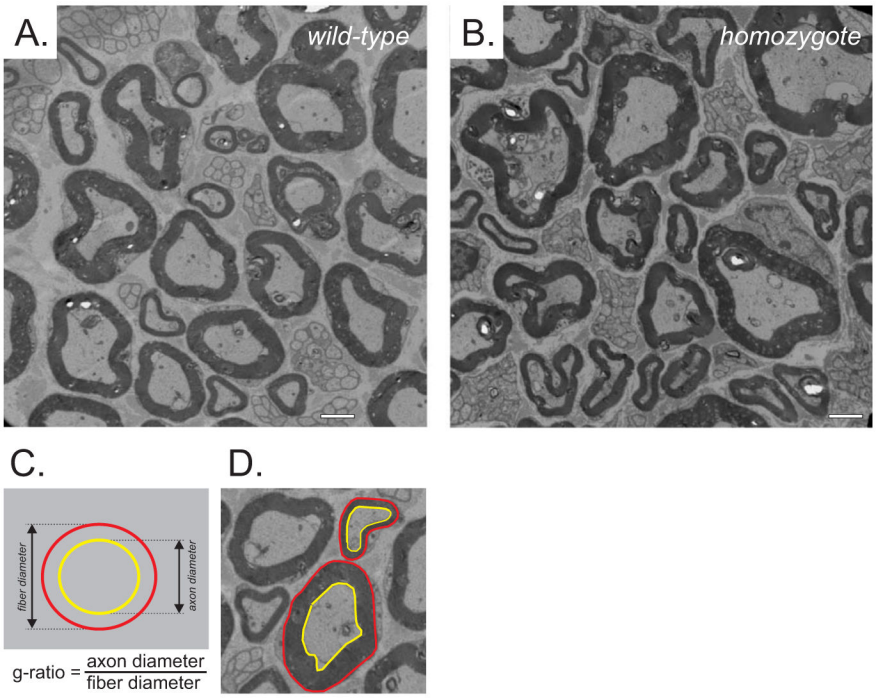

Fig. 2.

Electron micrographs of wild-type (A) and homozygous (B) sciatic nerves taken at a direct magnification of 4400x display no overt phenotype. Size bars = 2 μM. C: calculated g-ratio is the ratio of axon diameter divided by whole fiber diameter, it assumes the nerve fiber to be a “perfect” circle. D: g-ratio of actual nerve fibers is obtained by outlining the circumference of the axon (i.e. yellow in this case) and the circumference of the total fiber (i.e. red in this case). The outlines are done within Image J software, which allows for precise circumference measurements from which the diameters and g-ratio can be calculated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

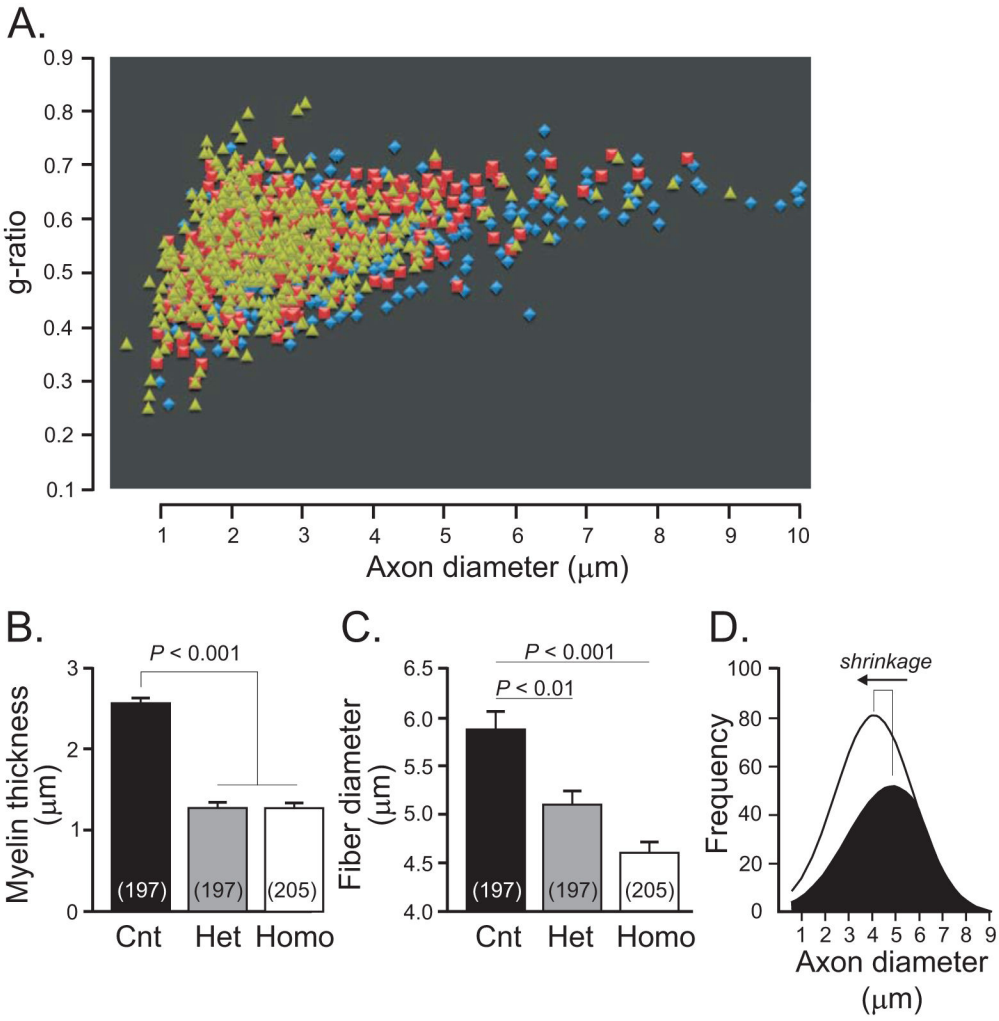

Fig. 3.

Shrinkage of axons and fibers in KCC3-T991A sciatic nerves. A: Graphing the axon diameter versus g-ratio reveals differences in spread (distribution) among homozygous (green triangles), heterozygous (red squares), and wild-type (blue diamonds) KCC3-T991A axon diameters. B: Myelin thickness is decreased in heterozygous and homozygous animals compared to controls ($P < .001$). C: When assessing total fiber diameter, both heterozygous and homozygous sciatic nerves displayed decreased diameters compared to controls. Bars represent mean ± S.E.M. (*n*, indicated within the bars). D: Distributions of sciatic nerve axon diameters of homozygous (white curve) and control (black curve) mice. A leftward shift of axon diameter (indicating shrinkage) is observed in the homozygous mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

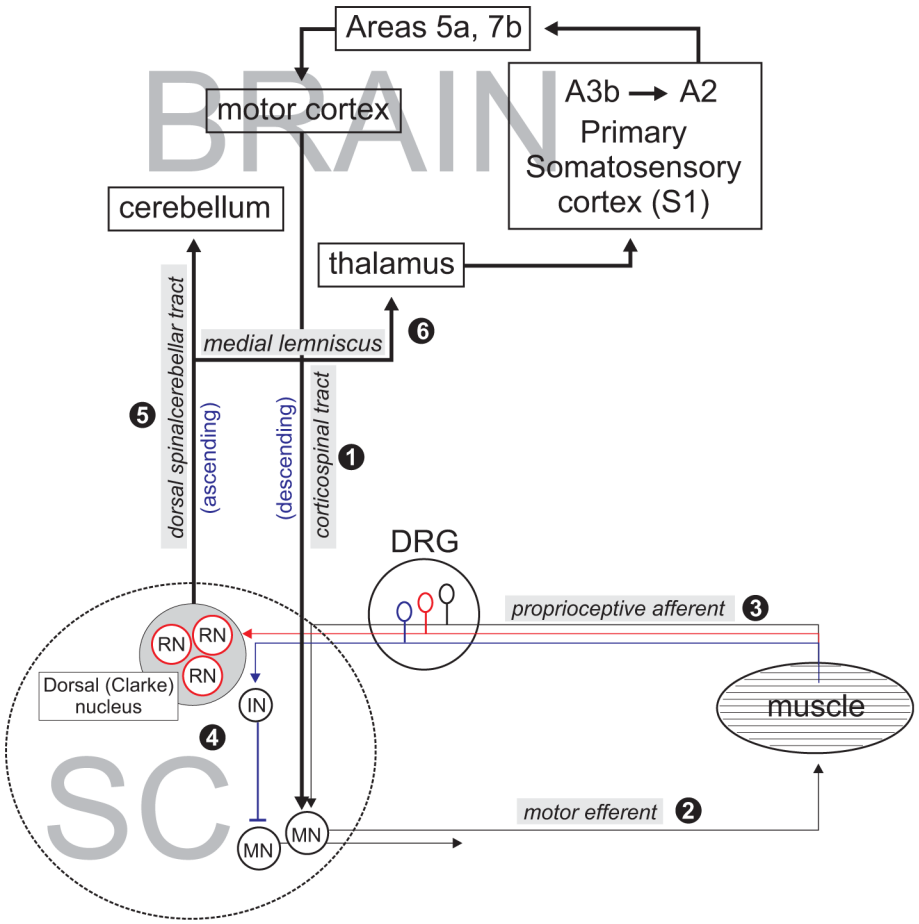

Fig. 4.

Structures of the PNS and CNS involved in locomotion. This connectivity diagram shows the flow of information within the spinal cord, muscles, and brain. (1) The motor cortex sends projections to the motor neurons via the corticospinal tract. (2) Motor neurons (MN) with their cell bodies in the ventral horn of the spinal cord send their axons to the periphery where they synapse onto muscles. (3) Sensory neurons, with their cell bodies in the dorsal root ganglia (DRG), bring proprioceptive information from muscles back to the spinal cord where they synapse onto interneurons (IN), relay neurons (RN), and motor neurons (MN). (4) IN synapse with some MN and inhibit them. (5) Some proprioceptive neurons, especially those from lower limbs, project to Clarke's nucleus in the lumbar spinal cord. Second order neurons from Clarke's nucleus then project to the cerebellum via the dorsal spinocerebellar tract. Some of these neurons give off collaterals (6) which join the medial lemniscus headed to the ventral posterior lateral nucleus (VPL) of the thalamus. The VPL projects to the primary somatosensory cortex (S1), which is composed of multiple Brodmann's areas. One area of S1, area 3b, carries proprioceptive information to Brodmann's area 2 within S1 before projecting to parietal areas 5a and 7b. Areas 5a and 7b then project to the motor and pre-motor cortex. Thus proprioceptive information can be used to influence voluntary motor movements.

Table 1

In the adult mouse CNS, KCC3 expression appears in multiple structures/regions. In the adult PNS, KCC3 expression has thus far been observed in DRG and nodes of Ranvier. Post-natal expression of KCC3 has been observed in the sciatic nerve and DRG, but KCC3 expression has not been studied extensively in post-natal PNS.

	PNS	CNS
Adult	Dorsal Root Ganglion (Byun and Delpire, 2007) Nodes of Ranvier- Schwann Cell (Sun et al., 2010)	Hippocampus, Choroid Plexus, Piriform Cortex, Cerebellum (Allen Brain Atlas, Rouzic et al., 2006) Brainstem, spinal cord (Allen Brain Atlas, Pearson et al., 2001) Hippocampal interneurons (Shekarabi et al., 2011)
Postnatal	Sciatic nerve, Dorsal root ganglia (Byun and Delpire, 2007)	

Table 2

Listed are all of the current mouse models of HSMN/ACC to date. Nearly all current mouse models except T991A observe the effects of a loss of function of KCC3.

References	KCC3 disruption	Behavior observed	Tissue pathology
Howard et al., 2002	Removal of exon 3: loss of function	- Dragging of hind limbs - Severe locomotor deficits - Defective paired pulse inhibition	- Hypomyelination - Axonal degeneration and swelling
Boettger et al., 2002	Disruption in open reading frame (exon 3): Global LOF of KCC3	- Severe locomotor deficits - Reduced seizure threshold - Deafness - Hypertensive	- Hypomyelination - Degenerating axons
Shekarabi et al., 2012	Deletion of Exon 18: Global LOF Exon 18: KCC3 LOF in synapsin 1-expressing neurons	- Dragging of hind limbs - Severe locomotor deficits	- Axon degeneration - Hypomyelination - Axonal swelling - Decreased brain masses
Ding and Delpire, 2014	Exon 7: KCC3 LOF in Parvalbumin neurons	- Severe locomotor deficits - Hyperactivity	Tissue vacuolization in DRG
	Exon 7: KCC3 LOF in NaV1.8 neurons	No abnormalities	No abnormalities
	Exon 7: KCC3 LOF in Schwann cells	No abnormalities	No abnormalities
	Exon 7: KCC3 LOF in Enolase-2 neurons (all neurons?)	No abnormalities	No abnormalities
Kahle et al., 2016	Exon 22: T991A Gain of Function (point mutation)	- Inability to maintain balance on rotarod and balance beam	- Swollen nerve pathology - Hypomyelination