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KCC3 loss-of-function contributes to Andermann syndrome by inducing activity-dependent neuromuscular junction defects

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ABSTRACT

Loss-of-function mutations in the potassium-chloride cotransporter KCC3 lead to Andermann syndrome, a severe sensorimotor neuropathy characterized by areflexia, amyotrophy and locomotor abnormalities. The molecular events responsible for axonal loss remain poorly understood. Here, we establish that global or neuron-specific KCC3 loss-of-function in mice leads to early neuromuscular junction (NMJ) abnormalities and muscular atrophy that are consistent with the pre-synaptic neurotransmission defects observed in patients. KCC3 depletion does not modify chloride handling, but promotes an abnormal electrical activity among primary motoneurons and mislocalization of Na^+/K^+-ATPase α1 in spinal cord motoneurons. Moreover, the activity-targeting drug carbamazepine restores Na^+/K^+-ATPase α1 localization and reduces NMJ denervation in *Slc12a6*$^{-/-}$ mice. We here propose that abnormal motoneuron electrical activity contributes to the peripheral neuropathy observed in Andermann syndrome.

1. Introduction

Andermann syndrome is an autosomal recessive neurodevelopmental and neurodegenerative disorder characterized by peripheral neuropathy with variable agenesis of the corpus callosum (ACCPN) (Larbrisseau et al., 1984; Mathieu et al., 1990), hypotonia and amyotrophy (DeBraekeleer et al., 1993) and is caused by mutations within the cation-chloride cotransporter KCC3 (Howard et al., 2002).

KCC3-deficient mice reproduce the typical ACCPN sensorimotor histophysiopathology, displaying impaired locomotor and sensorimotor gating capacities and decreased peripheral nerve conduction (Howard et al., 2002; Boettger et al., 2003; Sun et al., 2010; Shekarabi et al., 2012). Axonal swelling, neurodegeneration and hypomyelination of the sciatic nerve seem to account for the peripheral neuropathy. Both enveloping glial cells and demyelinating events have been proposed as the primary events responsible for reduced nerve conduction and neurodegeneration in mice and patients (Howard et al., 2002; Dupre et al., 2003; Sun et al., 2010). However, neuron-specific KCC3 deletion

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in mice is sufficient to induce peripheral neuropathy, supporting a neuronal-dependent neuropathy (Shekarabi et al., 2012).

KCC3 belongs to the family of cation-chloride cotransporters coded by the *Slc12a* genes (Mount et al., 1999). The *Slc12a* gene family comprises seven members coding for two inwardly directed Na^+-K^+-$2Cl^-$ cotransporters (*Slc12a2* for ubiquitous NKCC1 and *Slc12a1* for kidney specific NKCC2), one Na^+-Cl^- cotransporter (*Slc12a3* for kidney specific NCC) and four outwardly directed K^+-Cl^- cotransporters, (*Slc12a4–7* for KCC1–4, respectively) (Gagnon and Delpire, 2013). These membrane proteins are responsible for a wide variety of functions including cell volume regulation (O'Neill, 1999) and maintenance of intracellular chloride concentration $[Cl^-]_i$ (Blaesse et al., 2009). Following an osmotic challenge, KCCs extrude K^+ and Cl^- ions together with water. Concordantly, axon swelling and periaxonal fluid accumulation are observed early in postnatal day (P)3, P8 and P30 KCC3-depleted (*Slc12a6*$^{-/-}$) mice followed by axonal loss in 7–8 month old adult mice (Byun and Delpire, 2007). As younger P30 mice perform poorly motor tests without any apparent axonal degeneration, it is assumed that defects in volume regulation are major contributor to impaired motor and balance functions (Howard et al., 2002; Byun and Delpire, 2007). However, the potential impact of KCC3 loss-of-function on neuromuscular junction (NMJ) integrity, which could also account for early motor defects, has never been explored, nor have activity-dependent effects of KCC3 through its potential control on neuronal intracellular Cl^- and K^+ content (Boettger et al., 2003; Lucas et al., 2012).

Here, we show that axon denervation occurs at the NMJ in P30 *Slc12a6*$^{-/-}$ mice, therefore representing an early sign of the peripheral neuropathy. We further demonstrate that KCC3 loss-of-function does not modify the developmental chloride shift observed in spinal cord and isolated motoneurons. Instead, *Slc12a6*$^{-/-}$ motoneurons display an abnormal electrical activity, a possible consequence of an unbalanced ionic homeostasis during firing behaviour related to Na^+/K^+-ATPase dysfunction. Consistent with this proposed detrimental role on electrical activity, we found that a pharmacological decrease of motoneuron activity *in vivo* ameliorated NMJ innervation of *Slc12a6*$^{-/-}$ mice. Combined with the functional analysis of nerve-evoked muscle contraction in ACCPN patients, our results highlight pre-synaptic-dependent defects as playing a central part of the Andermann syndrome and identify a novel mechanism by which loss of KCC3 leads to motor symptoms in this neurodevelopmental disorder.

2. Material and methods

2.1. Animals

All animal experiments were approved by the national ethics committee on animal experimentation, and were done in compliance with the European community and national directives for the care and use of laboratory animals. *Slc12a6*$^{+/-}$ mice, described in (Howard et al., 2002) were maintained on a C57BL/6 background and we used the offspring of crosses between heterozygotes. For experiments using *nsSlc12a*$^{\Delta 18/\Delta 18}$ mice, described in (Shekarabi et al., 2012), we used tissues collected at McGill University.

For *in vivo* experiments, carbamazepine (CBZ) or vehicle was administered to P10 *Slc12a6*$^{-/-}$ mice by intraperitoneal injection of 0.025 mg/g/day for 20 consecutive days as described in (Li et al., 2013). P30 mice were then euthanized and muscles processed for neuromuscular junction analysis. A stock solution was prepared at 2.5 mg/ml using 12.5 mg CBZ (Santa-Cruz Biotechnology) diluted with 5 ml 2-Hydroxypropyl-β-cyclodextrin (Santa-Cruz Biotechnology) and stored at +4 °C.

2.2. Motoneuron culture

Genotyping was carried out by PCR on tail DNA of E12.5 embryos obtained from *Slc12a6*$^{+/-}$ mice breeding. Embryos were kept at 4 °C in Hibernate-E medium (Life technologies) during the course of genotyping. Afterwards, *Slc12a6*$^{+/+}$ and *Slc12a6*$^{-/-}$ embryos were processed for cultures as described previously (Raoul et al., 2002). Briefly, cells were dissociated mechanically after trypsin treatment of the dissected spinal cords. The largest cells were isolated using iodixanol density gradient purification. To get highly purified motoneuron cultures, we added an immunopurification step using magnetic cell sorting technology (Arce et al., 1999). In experiments using *Hb9::GFP* embryos to identify motoneurons, we found that this protocol yielded roughly 80–90% of GFP-positive neurons. Briefly, isolated neurons were incubated at +4 °C in 80 μl L-15 medium containing 0.5% bovine serum albumin (BSA) and 2 μl of an anti-mouse p75 monoclonal antibody (Millipore) for 20 min, followed, after wash-out, by a 15 min incubation in 80 μl L-15, 0.5% BSA, 10 μl goat anti-mouse IgG microbeads. The magnetically labeled cells were applied onto a separation column and retained using a magnet (Miltenyi Biotec). Following wash-out of the p75 negative fraction with L-15, 05% BSA, cell sorting was achieved by removing the magnet. After a final BSA cushion, motoneurons were plated onto poly-ornithine laminin-coated wells in Neurobasal (Life Technologies) medium containing 2% horse serum, 2% B-27 supplement (Life Technologies), 50 μM L-glutamine, 25 μM L-glutamate, 25 μM β-mercaptoethanol and a cocktail of neurotrophic factors (1 ng/ml BDNF, 100 pg/ml GDNF, and 10 ng/ml CNTF).

2.3. Electrophysiological recordings

For chloride reversal potential determination of 1 and 7 *days in vitro* (DIV) motoneurons, chloride $GABA_A$ current was recorded at 20–22 °C with the gramicidin-perforated patch-clamp technique using an Axopatch 200B amplifier (Dipsi Industrie). The bathing solution contained 140 mM TEA-Cl, 3.5 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.4 with CsOH, and the pipette solution contained 140 mM CsCl, 1.5 mM Mg-ATP, 0.5 mM Na-GTP, 0.1 mM EGTA and 10 mM HEPES, adjusted to pH 7.35. We determined $[Cl^-]_i$, by adding 50 μg/ml gramicidin A (Sigma-Aldrich) to the pipette solution as described in (Pieraut et al., 2007). $[Cl^-]_i$ was calculated according to the Nernst equation: $E_{rev} = RT/ZF \times (\log ([Cl^-]_i / [Cl^-]_e)$, where $RT/ZF =$ 58 mV at room temperature and $[Cl^-]_e = 147$ mM.

For electrical activity measurements, 7–8 DIV motoneurons from *Slc12a6*$^{+/+}$ and *Slc12a6*$^{-/-}$ were recorded under whole-cell patch clamp at room temperature in a bathing solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.35. The patch pipette contained 10 mM KCl, 135 mM K-methane-sulfonate, 1.5 mM Mg-ATP, 0.5 mM Na-GTP, 0.1 mM EGTA and 10 mM HEPES, adjusted to pH 7.35. Spontaneous activity of motoneurons was recorded with the loose-patch technique after forming a 30–50 MΩ contact between cell membrane and recording electrode. The electrode was filled with the extracellular solution. Ouabain, apamin and cadmium chloride were from Sigma.

2.4. RNA extraction and RT-qPCR

For primary cultures, total mRNA was extracted from roughly 30,000 purified embryonic motoneurons at 1 or 7 DIV with the RNeasy Mini Kit (Qiagen). For E12.5, P1 and P30 lumbar spinal cords, the tissues were harvested in RNA*later* stabilization buffer (Qiagen). Lysis buffer was used for pestle tissue crushing and homogenization by passing the lysate through needles. Lysates were then mixed with an equal volume of 70% ethanol, and total mRNA was separated from other cellular components on RNeasy minispin columns. The eluted mRNA was quantified by spectrophotometry (Nanodrop). Following gDNA wipe out, reverse transcription (RT) was performed with 100 ng to 1 μg of mRNA with the Quantitect RT kit (Qiagen). The collected cDNA was diluted to 50 ng (culture) or 100 ng (tissue) with water and stored at −20 °C until further use. Primers were designed with Primers 3.0 software. The sequences of the primers used are published in (Lucas et al., 2012).