BRIEF REPORT

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A Gain-of-Function Mutation in *NALCN* in a Child with Intellectual Disability, Ataxia, and Arthrogryposis

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ABSTRACT: *NALCN* and its homologues code for the ion channel responsible for half of background Na^+-leak conductance in vertebrate and invertebrate neurons. Recessive mutations in human *NALCN* cause intellectual disability (ID) with hypotonia. Here, we report a de novo heterozygous mutation in *NALCN* affecting a conserved residue (p.R1181Q) in a girl with ID, episodic and persistent ataxia, and arthrogryposis. Interestingly, her episodes of ataxia were abolished by the administration of acetazolamide, similar to the response observed in episodic ataxia associated with other ion channels. Introducing the analogous mutation in the *Caenorhabditis elegans* homologue *nca-1* induced a coiling locomotion phenotype, identical to that obtained with previously characterized *C. elegans* gain-of-function *nca* alleles, suggesting that p.R1181Q confers the same property to NALCN. This observation thus suggests that dominant mutations in *NALCN* can cause a neurodevelopmental phenotype that overlaps with, while being mostly distinct from that associated with recessive mutations in the same gene.

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KEY WORDS: NALCN; de novo; gain-of-function; *C. elegans*; Na^+-leak

Members of the 24-transmembrane domain ion channel superfamily play crucial roles in almost every excitable cell of the body. These channels form pores that are permeable to cations, including Na^+ and Ca^{2+}. They share a common architecture of four homologous domains (I–IV), consisting of six transmembrane helices (S1–S6) separated by three cytoplasmic linkers. The homologous domains participate in the formation of the pores, and establish the selectivity filters for ion permeability. One member of this family, NALCN (MIM# 611549), is a largely voltage-independent channel, which controls neuronal excitability in the mouse brain by contributing to the TTX-insensitive background Na^+ leak conductance [Lu, et al., 2007]. *Nalcn* null mice die shortly after birth, at least in part because of disrupted respiratory rhythm [Lu, et al., 2007], whereas loss-of-function mutations affect neuronal resting membrane potential, excitability, synaptic vesicle turnover, persistent motor circuit activity, and sustainability of locomotion in *Caenorhabditis elegans* and sustainability of locomotor activity in *Drosophila* [Lear, et al., 2005; Humphrey, et al., 2007; Jospin, et al., 2007; Yeh, et al., 2008; Lu, et al., 2010; Xie, et al., 2013; Gao, et al., 2015].

Recently, recessive mutations in *NALCN* were reported in children with global developmental delay and hypotonia from three consanguineous families [Al-Sayed, et al., 2013; Koroglu, et al., 2013]. In one of these families, affected siblings also show severe growth retardation and neuroaxonal dystrophy [Koroglu, et al., 2013]. Here, we describe the identification of a de novo missense mutation in *NALCN* in a child with intellectual disability (ID), episodic and persistent ataxia, and congenital arthrogryposis. Functional studies performed using the *C. elegans* model indicate that this mutation induces a gain-of-function phenotype, suggesting that dominant mutations in *NALCN* can also disrupt brain development in humans.

This study was prospectively reviewed and approved by the CHU Sainte-Justine research ethics committee and informed consent was obtained from the parents. The subject (1140.420), a 7.5 years old girl, is the first child of unrelated parents. The parents are healthy except for a history of carsickness in the mother. The subject was born after an uneventful pregnancy and normal term vaginal delivery with a weight of 3.2 kg and head circumference of 34.5 cm. She presented at birth with bilateral clubfeet and ulnar deviation and flexion contractures of the fingers in both hands.

The patient had a global developmental delay with axial and peripheral hypotonia. She started to sit on her own at 17 months, to stand with support at 18 months, and took her first steps at 23 months. She remained unstable with a wide-base gait since learning to walk. In this context, she was investigated for a suspected neuromuscular disorder. Her blood creatine kinase levels were normal. An electromyogram performed at 29 months of age revealed high-amplitude polyphasic potentials with reduced recruitment,

Additional Supporting Information may be found in the online version of this article.

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suggesting a neurogenic deficit. Motor conduction velocities were normal at the posterior tibialis, but sensory conduction velocities at the median nerve could not be interpreted because of artifacts. Biopsies of the sural nerve and quadriceps muscle were normal.

At 3.5 years of age, she developed intermittent episodes of malaise, diaphoresis, irritability, ataxia, and vomiting lasting 15–30 min, up to five times per week, triggered by fatigue, strong emotions, quick movements, or car travel. During those episodes, she would be unable to stand, would cry, and be in great distress. These episodes were initially thought to be migraine equivalents but were refractory to amitriptyline. They increased in frequency up to five times per day at age 4.5 years old. Episodic ataxia was suspected and treatment with acetazolamide completely abolished the episodes.

Currently, the patient remains with gait ataxia and frequent falls. She can walk short distances independently, but uses a walker for longer distances. She remains clumsy on fine motor skills: she cannot use utensils, dress, draw, or pile up blocks. Her communication skills are limited: she does not talk but she uses hand signs and pictograms for basic needs. She understands simple verbal orders. She has motor agitation and a short attention span. Her cognitive evaluation at 5 years of age revealed moderate intellectual deficiency with motor skills equivalent to 16 months, receptive language of 12–15 months, expressive communication skills of 6 months, information processing (non-verbal skills) of 24 months of age.

On examination, she had minor dysmorphisms including downslanting palpebral fissures, broad nasal bridge, large nares, short columella, and full lips (Fig. 1A). Her head circumference was at 48 cm (3rd percentile). She was joyful and had good eye contact. She displayed stereotypic hand movements. She had right eye esotropia. Visual pursuit was difficult to elicit and was characterized by ocular apraxia. Spontaneous eye movements were complete in all directions. She had upper limb hypotonia but mild spasticity on right foot dorsiflexion. Her reflexes were normal and symmetrical. She had axial and gait ataxia with mild bilateral dysmetria. She had fixed ulnar deviation and flexion contractures of the fingers in both hands (Fig. 1A).

Brain MRI performed at 11 months of age was normal but a subsequent MRI scan at 4.5 years revealed diffuse moderate cerebellar atrophy. Electroencephalography was normal. Chromosomal array hybridization did not show any abnormality. A comprehensive metabolic work-up (plasma glucose, lactate and ammonia measurements, plasma amino acid chromatography, plasma acylcarnitine profile, transferrin isoelectric focusing, and urine organic acid chromatography) was negative. The patient had electrocardiograms at the ages of 1 and 2.5 years, which were normal. Echocardiography revealed small atrial and ventricular septal defects at birth, which resolved by 1 year of age. Mutation analysis of the *MECP2* and *MYH3* genes as well as methylation studies of the region associated with the Angelman syndrome were also negative.

Exome capture was performed on blood genomic DNA from the proband and her parents using the Agilent SureSelect Human All Exon Capture Kit (V3, 50 Mb; Mississauga, ON), and sequenced on the Illumina HiSeq2000 (paired-end, 2 × 100 bp, 3 exomes/lane format) at the McGill University and Génome Québec Innovation Center (Montreal, Canada). Sequence processing, alignment, variant calling, and prioritization were done as previously described [Hamdan, et al., 2014]. The exome target base coverage for the trio was 125–130×, with 95% of the target bases being covered at ≥10×. Only the variants with a genotype quality ≥20, whose positions were covered at ≥10× and supported by at least four variant reads constituting ≥20% of the total reads for each called position, were retained. This resulted in 21,906 variants affecting the coding or consensus splice sites in the proband's exome. We further filtered out synonymous variants that are not predicted to affect splicing and variants present in >0.5% of our in-house exomes (n = 600) from unrelated projects, and /or having a minor allele frequency of >0.5% in the 1000 Genomes or in the Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS/) datasets. Among the 575 remaining rare variants, we did not identify any homozygous or Compound heterozygous variants in a given gene. After excluding rare variants inherited from the parents, we identified potential de novo variants in *NALCN* (chr13:101736103C>T, hg19 build: NM_052867.2:c.3542G>A [p.R1181Q]) and *CFP* (chrX:47486948G>A, hg19 build; NM_002621:c.496C>T [p.P166S]). Sanger sequencing in the proband and her parents confirmed that these 2 variants were indeed de novo (Fig. 1B).

CFP (MIM# 300383) encodes properdin, a plasma glycoprotein that regulates the alternative complement pathway of the innate immune system and whose mutations have been implicated in X-linked properdin deficiency resulting in high susceptibility to meningococcal infections [van den Bogaard, et al., 2000]. Because of the apparent lack of requirement of *CFP* for brain development or function, we did not further consider the variant in this gene as a candidate. The *NALCN* variant, not previously reported in public SNP databases (1000 Genomes, EVS, or dbSNP), affects a highly conserved amino acid residue present in the cytoplasmic linker connecting domains III to IV (Fig. 1B and C). This NALCN variant, which now has been deposited in ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/variation/188046/), is predicted to be damaging by SIFT (score 0.01; version1.03, SIFT/PROVEAN Human SNPs; http://sift.jcvi.org/) and possibly damaging by Polyphen-2 (score HumDiv 0.7; version 2.2.2r398; http://genetics.bwh.harvard.edu/pph2/).

Two homologues of human NALCN exist in *C. elegans* (NCA-1 and NCA-2) and function redundantly to affect locomotion [Humphrey, et al., 2007; Jospin, et al., 2007; Pierce-Shimomura, et al., 2008; Yeh, et al., 2008; Gao, et al., 2015]. We previously reported that the double deletion mutant of *nca-1* and *nca-2*, *nca-1(gk9);nca-2(gk5)*, fails to sustain sinusoidal locomotion, a phenotype called "fainter" (Supp. Movies S1 and S2) [Yeh, et al., 2008; Xie, et al., 2013; Gao, et al., 2015]. On the other hand, two gain-of-function alleles in NCA-1, *hp102* (p.R403Q; NP_741413.2) and *e625* (p.A717V; NP_741413.2), showed exaggerated body bends during movements, a phenotype called "coiler" (Supp. Movie S3) [Yeh, et al., 2008]. Wild-type *C. elegans* expressing a mouse *Nalcn* cDNA that carries the *hp102* equivalent mutation (*Nalcn* R329Q; NP_796367.3) in its nervous system exhibited a coiler phenotype, suggesting that the *C. elegans* and mammalian homologues can mediate similar physiological functions in *C. elegans* neurons [Yeh, et al., 2008].

The de novo mutation identified herein affects an amino acid (p.R1181) that is conserved both in NCA-1 (residue R1230; NP_741413.2) and NCA-2 (residue R1222; NP_498054.2) (Fig. 1C). To verify the genetic nature (recessive versus dominant) and impact (loss- versus gain-of-function) of the p.R1181Q mutation in vivo, we examined the effect of expressing *C. elegans* NCA carrying this mutation in *nca-1(gk9);nca-2(gk5)* fainters and the wild-type animals (Supp. Movies S4, S5, and S6). If R1230Q leads to functional loss, it should not rescue the fainter phenotype and should not alter the locomotion of wild-type animals. If R1230Q results in a dominant-negative effect, we should observe the fainter phenotype in both the double mutants and wild-type animals. Finally, if R1230Q leads to gain-of-function, it should convert both fainters and wild-type animals to coilers.

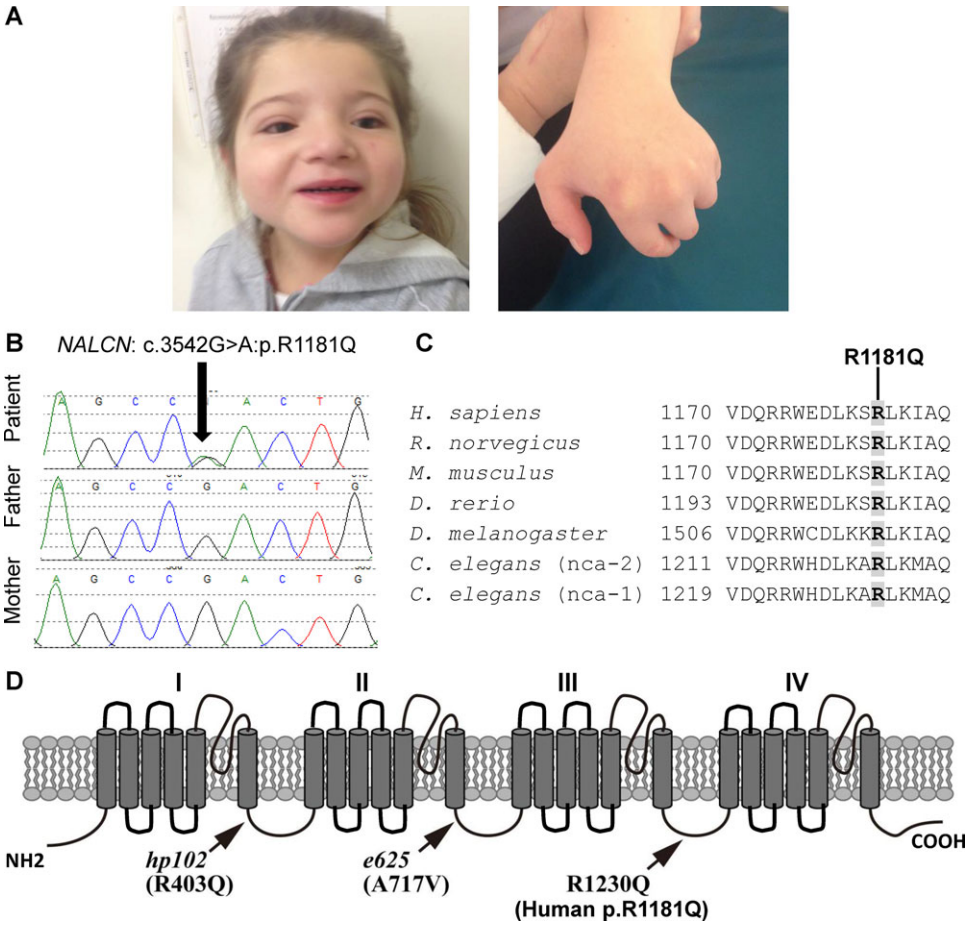

Figure 1. De novo mutation in *NALCN*. **A:** Pictures of the patient and of one of her hands. **B**: DNA sequence chromatograms showing the de novo mutation (c.3542G>A:p.R1181Q) in *NALCN* identified herein. Nucleotide numbering of the mutations herein reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the NCBI reference sequence NM_052867.2, whereas the amino positions are based on the corresponding NCBI reference sequence NP_443099.1. **C:** Homologene-generated (NCBI) amino acid alignment of part of human NALCN with its orthologues showing the conservation of the R1181 residue across the various species (*H. sapiens*, NP_443099.1; *R. norvegicus*, NP_705894.1; *M. musculus*, NP_796367.3; *D. rerio*, NP_001017549.2; *D. melanogaster*, NP_001096981.1, *C. elegans* (*nca-1*, NP_741413.2; *nca-2*, NP_498054.2)). **D**: Schematic diagram of the *C. elegan* nalcn (*nca-1*) protein showing the repeated domains (I–IV) each containing 6 transmembrane domains (S1–S6). Localization of the p.R1181Q mutation and relative positions of the published NCA-1 gain-of-function mutations (R403Q in the *hp102* mutant; A717V in the *e625* mutant) are also shown.

As we reported previously [Xie, et al., 2013], the pan-neuronal expression of a wild-type NCA-1 cDNA fully rescued the fainter phenotype in *nca-1(gk9);nca-2(gk5)* to that of wild-type animals (Fig. 2A; Supp. Movie S4). On the other hand, the pan-neuronal expression of NCA-1 cDNA carrying the R1230Q mutation in *nca-1(gk9);nca-2(gk5)* induced the same coiling locomotion identical to that of the gain-of-function *nca-1(hp102)* or *nca-1(e625)* mutants (Supp. Movie S5). The same transgenic array also produced a coiling phenotype in the wild-type background (Fig. 2A; Supp. Movie S6). Hence, R1230Q in *C. elegans* NCA-1 and its equivalent in human NALCN, p.R1181Q, induced a gain-of-function, dominant effect.

In addition to the motor pattern changes, we further compared the effect of loss-(fainters) and gain-(coilers)of-function of NCA on the physiology of *C. elegans* neurons. We examined the activity of the AVA premotor interneuron in moving animals by Ca^{2+} imaging. In wild-type animals, the calcium rise of AVA corresponded with the initiation of backward locomotion, peaking upon the cessation of backing (Fig. 2B). Similar to our previous report, in fainters, the calcium spikes exhibited by AVA were significantly reduced in amplitude and increased in frequency when compared with that of wild-type animals, consistent with the significantly reduced duration of backing events (Fig. 2B and C) [Xie, et al., 2013]. Coilers exhibited an interesting phenotype: they retained large calcium spikes and long backing comparable to those of wild-type animals, with additional, high incidence of small calcium spikes that corresponded with small backing events (Fig. 2B and C). These results suggest that gain-of-function alleles of NCA cause distinct motor behaviors from that of loss-of-function alleles, and also partially overlapping changes for neurophysiology.

The fact that the de novo mutation identified in our case confers a gain-of-function in the context of a well-validated in vivo paradigm strongly suggests that it is pathogenic. Both dominant and recessive mutations in *NALCN* would thus have the potential of disrupting neuronal function in humans. The phenotypes observed in our subject and in the cases with recessive mutations overlap to the extent that they all show severe global developmental delay with hypotonia. However, some cases with the recessive mutations also display neuroaxonal dystrophy, whereas our case had normal nerve biopsy devoid of spheroid bodies. By contrast, she was born with arthrogryposis and developed ataxia. The

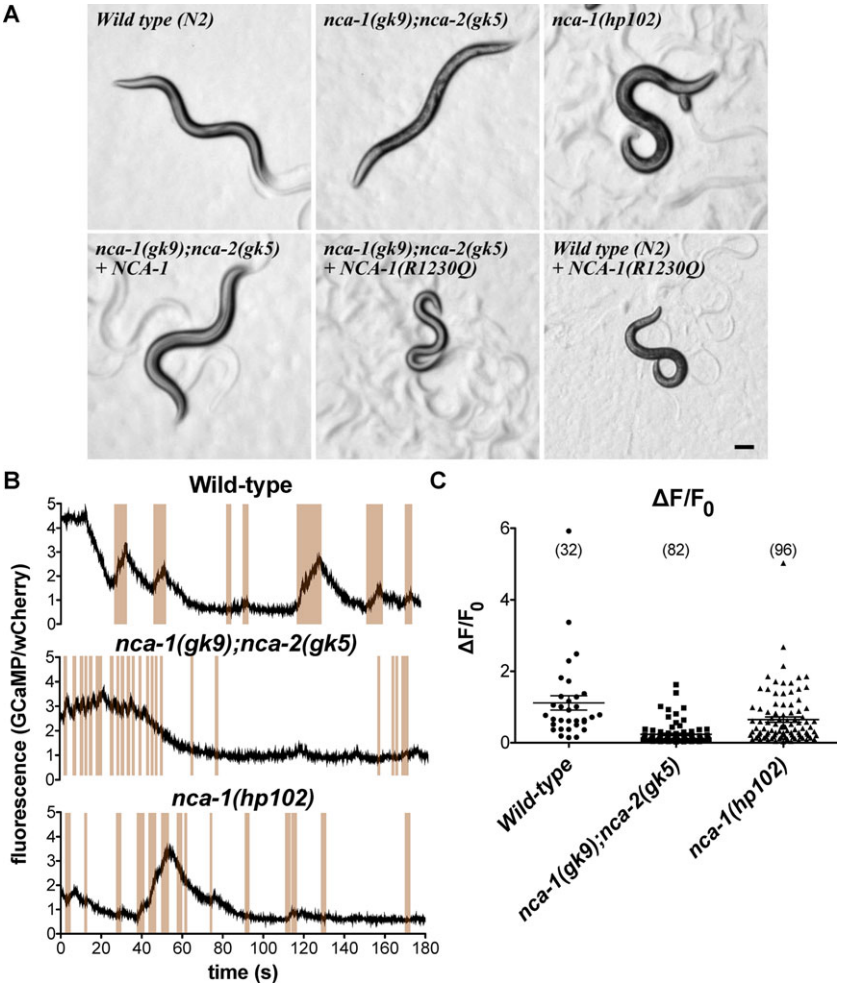

Figure 2. Gain-of-function and loss-of-function mutations in *C. elegans nca* modify animal motor patterns and neurophysiology. **A:** Snapshots of the body posture of wild-type (N2), *nca-1(gk9),nca-2(gk5)*, and *nca-1(hp102)* animals during locomotion. Wild-type animals generate continuous sinusoidal locomotion; *nca-1(gk9);nca-2(gk5)* is a fainter, with frequent pausing with a reduced body curvature, and *nca-1(hp102)* is a coiler, exhibiting exaggerated body curvature during movements. Neuronal expression of the wild-type NCA-1 cDNA converts the *nca-1(gk9);nca-2(gk5)* fainters to wild-type-like movements. Neuronal expression of NCA-1(R1230Q) converted both fainters and wild-type animals to *nca-1(hp102)*-like coilers. Pictures show the representatives of 3 (*nca-1(gk9);nca-2(gk5)* + NCA-1), 2 (nca-1(gk9);nca-2(gk5) + NCA-1(R1230Q)) and, the same transgenic lines crossed out of the *nca-1(gk9); nca-2(kg5)* background (wild-type (N2) + NCA-1 (R1230Q)) transgenic mutant lines, respectively. Scale bar: 0.1 mm. **B:** Distinct calcium profiles of the AVA premotor interneuron in moving animals in wild-type animals, fainters, and coilers. The black line shows the fluorescent signals of a genetic calcium sensor in AVA, which correlates with neuronal activity. Periods in which the animal was moving backward are highlighted with a brown box. In all genotype, the calcium rise in AVA was observed during the backing events. In the *nca-1(gk9);nca-2(gk5)* fainters, the short backing events and small AVA calcium peaks replaced the long backing and large calcium spikes that were observed in wild-type animals. The *nca-1(hp102)* coilers exhibited large calcium spikes, as well as small events. 10–14 animals were imaged per genotype. **C:** Quantification of the peak amplitude of all calcium spikes ($\Delta F/F_0$) in wild-type animals, fainters, and coilers. Each data point represents a single calcium peak. The peaks were quantified from 8 to 14 animals per genotype. *** $P < 0.0001$; ** $P = 0.0077$, by unpaired Student's *t*-test. Error bars, SEM.

overlapping phenotypes caused by gain-of-function and loss-of-function mutations in disease-related genes have been noted in other neurodevelopmental disorders, including in Angelman syndrome and Rett syndrome (reviewed in [Ramocki and Zoghbi, 2008]).

While this manuscript was in revision, Chong et al. (2015) reported the identification of 14 de novo missense mutations in *NALCN* in individuals who showed a similar phenotype as that of our case, including developmental delay/ID with hypotonia and congenital contractures of the limbs [Chong, et al., 2015]. Moreover, one of these mutations, p.R1181Q, was the same as that found in our case. All together, these observations strongly support our conclusion that this mutation is pathogenic. Although some of the subjects described by Chong et al. (2015) had cerebellar atrophy on their MRI, the authors did not report that they were ataxic. It will be important to determine whether ataxia is a common but overlooked feature in patients with de novo mutations in *NALCN* or whether it represents an atypical manifestation of this condition.

The p.R1181Q mutation and the gain-of-function mutations previously characterized in *C. elegans* are all located in the cytoplasmic interdomain linkers (see Fig. 1D). The homologous regions in other members of the 24-transmembrane domain family have been involved in the regulation of channel activity [Cantrell and Catterall, 2001]. For instance, fast inactivation of voltage-gated Na^+ channels involves channel block by the III–IV cytoplasmic interdomain linker (reviewed in [Ulbricht, 2005]. The p.R1181Q mutation is located in the corresponding region of NALCN. Although it is unknown

whether NALCN undergoes fast inactivation, it is possible that this region also plays a regulatory role in NALCN and that the p.R1181Q mutation induces a gain-of-function by impairing negative gating of the channel. In contrast, most of the other mutations described by Chong et al. [2015] are located in pore-forming segments of the protein [Chong, et al., 2015]. These authors studied the functional impact of two of these mutations (p.Y578S and p.L590F), located in the transmembrane S6 of domain II, by performing co-transfection experiments in HEK293T cells [Chong, et al., 2015]. They found that the expression of the mutant NALCN proteins nearly abolished the expression of wild-type NALCN, suggesting a dominant negative effect. However, caution should be applied when interpreting transfection experiments performed in heterologous cell systems, as it is unclear whether the NALCN complex is correctly assembled and expressed at physiological levels in such cells. Nevertheless, it is possible that dominant mutations in *NALCN* affect the function of the channel through different mechanisms with mutations in the pore-forming segments causing a dominant-negative effect and mutations in the cytoplasmic interdomain linkers inducing a gain-of-function. It will be interesting to determine whether ataxia is found mainly in patients with mutations in the linker domains, suggesting that this feature is related to the gain-of-function.

Our case presented with an interesting constellation of clinical features, which can be attributed to dysfunction in various neuronal networks. NALCN is expressed in multiple brain regions in mammals (Allen Brain Atlas; http://www.brain-map.org/), including the dentate gyrus and pyramidal cell layer of the hippocampus, where its dysfunction may result in cognitive deficits. Indeed, overexpression of NALCN leads to a +20 mV increase in resting membrane potential and neuronal hyperexcitability in hippocampal neurons [Lu, et al., 2007]. This might in turn lead to perturbations in hippocampal network oscillations, altered signal processing and might result in learning deficits. NALCN is also highly expressed in the granular and Purkinje cell layers as well as in the dentate nucleus of the cerebellum. Increased excitability in these cell-types may cause ataxia by altering the burst-firing properties of granule cells or by erroneously enhancing output from cerebellar nuclei. One remarkable feature of the subject described here is that her episodic ataxia responded dramatically to the administration of acetazolamide, a carbonic anhydrase inhibitor. This is similar to the therapeutic response observed in patients with episodic ataxia type II due to mutations in *CACNA1A* encoding a voltage-gated calcium channel of the 24-transmembrane domain family. Although the precise mechanism of acetazolamide response in episodic ataxia is uncertain [Spacey, et al., 2004], one interesting possibility is that it also acts as a BK channel activator [Tricarico, et al., 2004]. In the context of a gain of NALCN function, activation of BK channels would be expected to hyperpolarize the resting membrane potential and counteract the gain in Na^+ leak currents. Additionally, verapamil, a calcium channel blocker approved for the treatment of migraines, arrhythmias and hypertension, has also been shown to inhibit the background Na^+ leak conductance associated with NALCN in neurons, and might be considered for the treatment of individuals with gain-of-function mutations in *NALCN*.

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