Neurobiology of Disease 115 (2018) 29–38

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Direct evidence of impaired neuronal Na/K-ATPase pump function in alternating hemiplegia of childhood

Christine Q. Simmons[a,1], Christopher H. Thompson[a,1], Bryan E. Cawthon[b], Grant Westlake[b], Kathryn J. Swoboda[c], Evangelos Kiskinis[d,e], Kevin C. Ess[b], Alfred L. George Jr[a,*]

[a] Department of Pharmacology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA
[b] Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN, USA
[c] Center for Genomic Medicine, Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
[d] Department of Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA
[e] Department of Physiology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

ARTICLE INFO

Keywords:
ATP1A3
Alternating hemiplegia of childhood
Induced pluripotent stem cells
Ion transporter

ABSTRACT

Mutations in *ATP1A3* encoding the catalytic subunit of the Na/K-ATPase expressed in mammalian neurons cause alternating hemiplegia of childhood (AHC) as well as an expanding spectrum of other neurodevelopmental syndromes and neurological phenotypes. Most AHC cases are explained by de novo heterozygous *ATP1A3* mutations, but the fundamental molecular and cellular consequences of these mutations in human neurons are not known. In this study, we investigated the electrophysiological properties of neurons generated from AHC patient-specific induced pluripotent stem cells (iPSCs) to ascertain functional disturbances underlying this neurological disease. Fibroblasts derived from two subjects with AHC, a male and a female, both heterozygous for the common *ATP1A3* mutation G947R, were reprogrammed to iPSCs. Neuronal differentiation of iPSCs was initiated by neurogenin-2 (NGN2) induction followed by co-culture with mouse glial cells to promote maturation of cortical excitatory neurons. Whole-cell current clamp recording demonstrated that, compared with control iPSC-derived neurons, neurons differentiated from AHC iPSCs exhibited a significantly lower level of ouabain-sensitive outward current ('pump current'). This finding correlated with significantly depolarized potassium equilibrium potential and depolarized resting membrane potential in AHC neurons compared with control neurons. In this cellular model, we also observed a lower evoked action potential firing frequency when neurons were held at their resting potential. However, evoked action potential firing frequencies were not different between AHC and control neurons when the membrane potential was clamped to −80 mV. Impaired neuronal excitability could be explained by lower voltage-gated sodium channel availability at the depolarized membrane potential observed in AHC neurons. Our findings provide direct evidence of impaired neuronal Na/K-ATPase ion transport activity in human AHC neurons and demonstrate the potential impact of this genetic defect on cellular excitability.

1. Introduction

Alternating hemiplegia of childhood (AHC) is a neurodevelopmental disorder caused by heterozygous, usually de novo, mutations in *ATP1A3* encoding the catalytic subunit of the neuronal ouabain-sensitive Na/K-ATPase complex (Heinzen et al., 2014; Rosewich et al., 2017). While AHC has been recognized as a distinct entity for many years (Verret and Steele, 1971; Krageloh and Aicardi, 1980), the genetic cause of the disorder was only recently discovered (Heinzen et al., 2012; Rosewich et al., 2012; Ishii et al., 2013).

AHC typically presents in infancy with paroxysmal hemiplegia, dystonia and abnormal ocular movements (Sweney et al., 2009; Panagiotakaki et al., 2010; Tenney and Schapiro, 2010; Tatli et al., 2011). Over time, independent involvement of both sides of the body, and migrating weakness or dystonia with bulbar, facial and limb involvement may occur. Episodes last for minutes to hours, terminated by either natural sleep or pharmacologically-induced sedation or anesthesia. Patients may appear to have seizures or stroke, but over time,

Abbreviations: AHC, alternating hemiplegia of childhood; RMP, resting membrane potential; iPSC, induced pluripotent stem cells; NGN2, neurogenin-2; RFP, red fluorescence protein; TetO, tetracycline inducible promoter; AHP, afterhyperpolarization

* Corresponding author at: Department of Pharmacology, Northwestern University Feinberg School of Medicine, Searle 8-510, 320 East Superior Street, Chicago, IL 60611, USA.
E-mail address: al.george@northwestern.edu (A.L. George).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.nbd.2018.03.009
Received 27 January 2018; Received in revised form 23 February 2018; Accepted 17 March 2018
Available online 19 March 2018

the pattern of recurrent hemiplegia with subsequent recovery, and the lack of epileptiform activity on EEG in conjunction with prolonged episodes, provides clues to the correct diagnosis. Most patients manifest developmental delay and intellectual disability, and approximately 50% ultimately manifest seizures, with onset months to years following onset of the initial movement disorder.

Current treatments are largely symptomatic and include sleep-inducing agents such as benzodiazepines or chloral hydrate to terminate prolonged episodes of hemiplegia or dystonia. Flunarizine, a sodium and calcium channel blocker with additional antihistamine effects used for migraine prophylaxis and less frequently for vertigo or adjuvant treatment for epilepsy, appears to reduce the frequency and/or duration of recurrent episodes of neurologic dysfunction in some AHC patients (Casaer and Azou, 1984; Casaer, 1987; Silver and Andermann, 1993; Sasaki et al., 2001). More targeted therapeutic strategies have been difficult to conceive without more information about the primary cellular and molecular defects underlying AHC.

In this study, we investigated the cellular consequences of one of the three most common, recurrent *ATP1A3* mutations associated with AHC using patient-specific induced pluripotent stem cell (iPSC) derived neurons. We determined that neurons differentiated from AHC patient-specific iPSCs exhibit lower levels of ouabain-sensitive outward current ('pump current') and depolarized potassium equilibrium potential predicting lower intracellular potassium ion concentration. These findings correlated with significantly depolarized resting membrane potential, and altered excitability observed in AHC neurons. Our findings provide direct evidence of impaired ion transport function of neuronal Na^+/K^+-ATPase and affirm loss-of-function as the primary molecular consequence of an AHC-associated *ATP1A3* mutation.

2. Materials and methods

2.1. Study subjects

Two unrelated subjects diagnosed with AHC were used in this study. Subject 44,629 was a female diagnosed with AHC at age 11 months. She experienced hemiplegic episodes approximately two times per month with each episode lasting 3–5 days, and has developmental delay. Genetic testing revealed the previously reported *ATP1A3* missense mutation c.2839G > A (p.G947R). The second subject (46337) was a male diagnosed with AHC after presenting at age 6 months with paroxysmal abnormal eye movements, alternating hemiplegia and dystonia. Other neurologic symptoms included balance problems, gait ataxia, cognitive impairment, visual loss, and migraines. At age 2 years, he walked normally but at age 10 years began having difficulty walking and then developed permanent quadriparesis at 19 years of age. Genetic testing identified *ATP1A3* c.2839G > A (p.G947R). Male and female healthy control subjects were previously described (Tidball et al., 2016).

2.2. Generation and validation of induced pluripotent stem cells

Primary fibroblast cultures were obtained from AHC patients or control subjects after informed consent using a protocol approved by the Institutional Review Board of Vanderbilt University Medical Center or the University of Utah. Skin biopsy was performed on subject 42,103 at age 16 months and on subject 46,337 at age 34 years. After expansion, fibroblasts were electroporated using the Neon Transfection System (Life Technologies, Carlsbad, CA) with episomal plasmids pCXLE-hOCT3/4-shp53-F, pCXLE-hSK and pCXLE-hUL described originally by Yamanaka and colleagues (Addgene plasmids 27077, 27078, 27079) (Okita et al., 2011). Approximately 2–3 weeks after transfection, nascent iPSC colonies were picked and replated on Matrigel-coated culture dishes using human embryonic stem cell media supplemented with ROCK inhibitor. The following day, the media was changed to a mixture of 75% human embryonic stem cell media and 25% mTeSR1 (StemCell Technologies, Vancouver, BC) without ROCK inhibitor. Three days after plating, media was switched to 50% mTeSR1 and 50% human embryonic stem cell media. On the days following, 25% of human embryonic stem cell media was replaced with 25% mTeSR1 media until reaching 100% mTeSR1 media. For each AHC fibroblast line, multiple clonal iPSC lines were generated and expanded.

Each clonal iPSC line was tested for expression of multiple stem cell markers including NANOG, OCT4, TRA1-60, SSEA-3 and SSEA-4. To verify pluripotency, all iPSC lines were differentiated using embryoid body protocols to generate and validate ectoderm, endoderm, and mesoderm lineage cell types (Shevde and Mael, 2013). Karyotypes were analyzed by a clinical cytogenetics laboratory (Genetics Associates, Inc., Nashville, TN). We verified the presence of heterozygous *ATP1A3* mutations in the AHC patient-specific iPSC lines using Sanger sequencing of PCR amplicons generated with exon-flanking primers, and also demonstrated for each iPSC line that plasmids encoding reprogramming factors had not integrated into the host cell genomes (primer sequences available upon request).

2.3. Neuronal differentiation from human iPSCs

Human iPSC-derived cortical excitatory neurons were generated by neurogenin-2 (NGN2) induction with modifications (Zhang et al., 2013). Briefly, human iPSCs in suspension were transduced by separate lentiviral vectors encoding neuogenin-2 driven by a tetracycline-inducible promoter (TetO-NGN2), reverse tetracycline-controlled transactivator (rtTA) and red fluorescent protein (RFP) then plated on matrigel-coated plates in E8 medium (Burridge et al., 2015) containing 10 μM Y27632 (StemCell technologies). Cells were maintained in a transitional medium from knockout serum replacement to neural induction medium with the supplements LDN-193189 (100 nM, Stemgent, Lexington, MA), SB431542 (10 μM, Stemgent), and XAV939 (2 μM, Sigma-Aldrich, St. Louis, MO). NGN2 and RFP expression were induced by 2 μg/ml doxycycline one day prior to a 48 h selection in puromycin (2 μg/ml). After day 5, induced neuronal cells were plated over mouse glial cells cultured on poly-D-lysine/laminin coated coverslips, and continually maintained in Neurobasal medium supplemented with N2, B27, BDNF (10 ng/ml, PeproTech, Rocky Hill, NJ) and doxycycline (2 μg/ml) for 21 to 25 days. Differentiated neurons were identified by morphology and red fluorescence then used for electrophysiology studies.

2.4. Immunofluorescent microscopy on differentiated neurons

Neuronal maturation and *ATP1A3* expression were assessed by immunofluorescent microscopy on differentiated excitatory neurons cultured for 21 to 25 days. Cells were fixed in 4% paraformaldehyde at room temperature for 15 min. After washing with phosphate-buffered saline (PBS) three times, the cells were permeabilized in 0.2% Triton X-100 in PBS for 10 min and blocked with 5% normal donkey serum for one hour. Primary antibodies were diluted 1:200 in the blocking solution and incubated at 4 °C overnight. The following primary antibodies were used: anti-ATP1A3 (XVIF9-G10; Thermo-Fisher, Waltham, MA), anti-NeuN (MAB377; EMD Millipore, Billerica, MA), and anti-tubulin (MAB1637; EMD Millipore). Cells were washed in PBS with 0.05% Triton X-100, and incubated with secondary Alexa Fluro-488 goat anti-mouse antibody (1:1000) followed by additional washes. Images were acquired using Nikon $C2^+$ confocal microscope in the Center for Advanced Microscopy (Feinberg School of Medicine).

2.5. Electrophysiology

2.5.1. Measurement of pump current

Whole-cell voltage-clamp recordings were made at room temperature using an Axopatch 200B amplifier (Molecular Devices). Patch pipettes were pulled from borosilicate glass capillaries (Warner