# A functional correlate of severity in alternating hemiplegia of childhood ☆,☆☆

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## ARTICLE INFO

## ABSTRACT

*Objective:* Mutations in *ATP1A3*, the gene that encodes the α3 subunit of the Na$^+$/K$^+$ ATPase, are the primary cause of alternating hemiplegia of childhood (AHC). Correlations between different mutations and AHC severity were recently reported, with E815K identified in severe and D801N and G947R in milder cases. This study aims to explore the molecular pathological mechanisms in AHC and to identify functional correlates for mutations associated with different levels of disease severity.

*Methods:* Human wild type *ATP1A3*, and E815K, D801N and G947R mutants were expressed in *Xenopus laevis* oocytes and Na$^+$/K$^+$ ATPase function measured. Structural homology models of the human α3 subunit containing AHC mutations were created.

*Results:* The AHC mutations examined all showed similar levels of reduction in forward cycling. Wild type forward cycling was reduced by coexpression with any mutant, indicating dominant negative interactions. Proton transport was measured and found to be selectively impaired only in E815K. Homology modeling showed that D801 and G947 lie within or near known cation binding sites while E815 is more distal. Despite its effect on proton transport, E815K was also distant from the proposed proton transport route.

*Interpretation:* Loss of forward cycling and dominant negativity are common and likely necessary pathomechanisms for AHC. In addition, loss of proton transport correlated with severity of AHC. D801N and G947R are likely to directly disrupt normal Na$^+$/K$^+$ binding while E815K may disrupt forward cycling and proton transport via allosteric mechanisms yet to be elucidated.

## Introduction

Alternating hemiplegia of childhood (AHC) is a neurological disorder characterized by repeated attacks of paralysis on one or both sides of the body beginning before 18 months of age. There is a pressing need for effective AHC treatment. Flunarizine, a non-selective calcium channel blocker, is a widely prescribed drug for AHC. Unfortunately, flunarizine only provides symptomatic relief and its efficacy varies between patients (Mikati et al., 2000; Neville and Ninan, 2007; Sweney et al., 2009). The development of AHC therapeutics has been hindered by its complex clinical presentation. There is a well recognized clinical heterogeneity in AHC, where some patients have longer or more frequent hemiplegic attacks in addition to greater extent of cognitive dysfunction and presence of co-morbidities such as seizure and respiratory complications. In contrast, some patients have relatively mild developmental delay and with few or no co-morbidities (Mikati et al., 2000; Sasaki et al., 2014; Yang et al., 2014).

Sequencing studies identified mutations in the gene, *ATP1A3*, as a primary cause of AHC (Heinzen et al., 2012; Ishii et al., 2013; Rosewich et al., 2012). Three recurring mutations within the gene account for ~60% of all AHC cases, D801N, E815K, and G947R. Furthermore, genotype-phenotype analysis revealed that these mutations correlated with clinical severity. In general, patients with D801N or G947R have better clinical outcomes than patients with E815K (Sasaki

et al., 2014; Yang et al., 2014). Because the genetic correlation with disease severity is strong, environmental factors are unlikely to play a major role in determining severity. The molecular and functional mechanisms responsible for this clinical heterogeneity are unknown.

*ATP1A3* encodes for the α3 subunit of the Na$^+$/K$^+$ ATPase. The α3 subunit is neuron specific, and is highly expressed in the cortex, hippocampus, basal ganglia and thalamus (McGrail et al., 1991). The α3 subunit has 10 transmembrane α-helices which contain the Na$^+$ and K$^+$ binding sites and the cytoplasmic domains involved in ATP hydrolysis (Bublitz et al., 2010). The majority of AHC mutations identified are located within the transmembrane helices (>70%) (Heinzen et al., 2012). Na$^+$/K$^+$ ATPase critically regulates the Na$^+$ and K$^+$ electrochemical gradients via forward cycling. Forward cycling describes the process by which Na$^+$/K$^+$ ATPase uses ATP hydrolysis to transport three Na$^+$ out and two K$^+$ into the cell (Post et al., 1972). Recently, the Na$^+$/K$^+$ ATPase has also been shown to conduct protons under physiologically relevant conditions. It is proposed that while Na$^+$ ions are leaving the Na$^+$/K$^+$ ATPase during forward cycling, an aqueous path is exposed, which allows protons to passively enter the cell (Vedovato and Gadsby, 2014). This newly revealed function is well positioned to impact neuronal excitability on account of well documented effects of intracellular protons on ion channels and receptors (Church et al., 1998; Takahashi and Copenhagen, 1996; Tombaugh and Somjen, 1996; Traynelis and Cull-Candy, 1991; Waldmann and Lazdunski, 1998).

The functional impact of *ATP1A3* mutations on Na$^+$/K$^+$ ATPase have been examined in model systems. Protein blots showed that mutations do not alter α3 subunit membrane expression while enzymatic assays found significant reductions in ATPase and phosphorylation activities, critical steps for proper forward cycling (Heinzen et al., 2012; Weigand et al., 2014). However, the extent of reduction in ATPase and phosphorylation activity was similar between mutations associated with mild and severe AHC. The binding capacity to ouabain, a Na$^+$/K$^+$ ATPase inhibitor, was also examined. Although D801N showed normal ouabain binding capacity it was absent in G947R and E815K (Weigand et al., 2014) and, importantly, no correlation with the disease severity was observed.

On the strength of genetic findings, this study hypothesized that the biophysical changes caused by individual AHC mutations are responsible for the correlations with AHC severity. Human mutations D801N, G947R and E815K, were expressed in *Xenopus laevis* oocytes and examined using electrophysiological techniques. The properties examined were forward cycling, dominant negativity and proton transport. Homology models of the human α3 subunit were also created to predict the structural-functional impact of mutations. A better understanding of *ATP1A3* mutations implicated in AHC may improve clinical diagnosis and prognosis and also revealing novel therapeutic approaches.

## Materials and methods

### Plasmid preparation

*ATP1A3* mutations examined in the human α3 subunit (Heinzen et al., 2012) were: c.2401G > A (D801N), c.2839G > C (G947R) and c.2443G > A (E815K). *Xenopus laevis atp1b3* was synthesized by Genscript (Piscataway, NJ). Since *Xenopus laevis* oocytes have endogenous *atp1b3*, *Xenopus laevis atp1b3* was used to avoid creating additional heterogeneity of assembled Na$^+$/K$^+$ ATPases which would have reduced the power to discriminate between various genotypes. All coding sequences were subcloned into an oocyte high expression vector (Liman et al., 1992) between restriction sites AccI and Bpu10I for *ATP1A3* and HindIII and BamHI for *atp1b3*. To minimize endogenous oocyte Na$^+$/K$^+$ ATPase activity, mutations which were known to reduce ouabain sensitivity, Q108R and N119D (Jewell and Lingrel, 1991), were introduced to the human α3 subunit by site directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent Technologies, Santa-Clara, CA). Na$^+$/K$^+$ ATPase with reduced ouabain sensitivity can be inhibited by high ouabain concentration (10 mM), but not by

low ouabain concentration (10 μM) (Vedovato and Gadsby, 2010). cDNAs were transcribed into capped cRNA *in vitro* (mMessage mMachine, Ambion, Austin, TX).

### Oocyte preparation and injection

Mature oocytes (Stage V or VI) were obtained from *Xenopus laevis*. Oocytes were defolliculated and isolated as previously described (Petrou et al., 1997). Oocytes were injected on the automated Roboinject platform (Multichannel Systems, Reutlingen, Germany). Each oocyte was injected with 15 to 30 ng of cRNA encoding for the human α3 subunit and 10 ng of cRNA encoding for the *Xenopus laevis* β3 subunit. Total injection volume was 50 to 100 nl. Oocytes were stored in ND96 solution at 17 °C for 3 days before recording.

### Two electrode voltage clamp recording

Before recording, oocytes were incubated in loading solution for 2 hours at 17 °C to increase intracellular Na$^+$. The loading solution contained: (mM) 95 NaOH, 90 sulfamic acid, 5 HEPES, 10 TEA-Cl and 0.1 EGTA, pH 7.5. Two electrode voltage clamp recording was performed on the automated Roboocyte2 platform (Multichannel Systems, Reutlingen, Germany). Oocytes were impaled with electrodes that contained 1.5 M K-acetate and 0.5 M KCl and were held at −50 mV. Voltage dependence was determined by measuring current in a series of 400 ms step from −140 to +40 mV every 2 s in 20 mV increments. Recording solution used to measure forward cycling contained: (mM) 115 NaOH, 110 sulfamic acid, 10 HEPES, 5 BaCl$_2$, 1 MgCl$_2$ and 0.5 CaCl$_2$, pH 7.5, as well as 10 μM ouabain to inhibit endogenous oocyte Na$^+$/K$^+$ ATPase. Forward cycling was activated by 15 mM of K-sulfamate. Recording solution used to measure proton transport was Na$^+$ and K$^+$ free, the NaOH was replaced with an equal concentration of TMA-OH. Responses were quantified at the end of the test pulse. All responses were subtracted from the background to ensure responses observed were from exogenous Na$^+$/K$^+$ ATPase. The background response was determined by adding 10 mM ouabain to recording solution, which would inhibit all Na$^+$/K$^+$ ATPase, including the ouabain insensitive Na$^+$/K$^+$ ATPase (Poulsen et al., 2010; Vedovato and Gadsby, 2010). Sampling frequency was 1 kHz and recording temperature was 20–22 °C.

### Homology modeling

Models of Na$^+$ and K$^+$ bound states of the human α3 subunit of the Na$^+$/K$^+$ ATPase were made using the structure of Na$^+$/K$^+$ ATPases from Pig Kidney (Kanai et al., 2013) (PDB accession code 3WGU) and shark rectal glands (Shinoda et al., 2009) (PDB accession code 2ZXE), sharing 88 and 86 % sequence identity to the human transporter respectively. Homology models were created by first aligning the sequences using Clustal W (Larkin et al., 2007), and using Modeller (Eswar et al., 2006) to generate and refine the structural model.

### Data analysis

Data was exported using the Roboocyte2+ software (Multichannel Systems, Reutlingen, Germany) and analyzed using AxoGraph (AxoGraph Scientific, Sydney, Australia). Statistical analysis was performed on GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Student's *t*-test and one-way ANOVA were used to test for statistical significance. Statistical significance was set at $p < 0.05$.

## Results

### Loss of forward cycling function with AHC mutations

Forward cycling was examined in oocytes expressing the wild type or AHC mutant constructs. Forward cycling current traces were first