Alternating Hemiplegia of Childhood Foundation

Research Project Grant Progress Report

August 12, 2013

Title: Molecular Physiology and Pharmacology of *ATP1A3* Mutations in AHC

Principal Investigator: Alfred L. George, Jr., M.D., Vanderbilt University

Co-investigator: Kevin C. Ess, M.D., Ph.D., Vanderbilt University

Project Funding Period: November 1, 2012 - July 31, 2013

Signatures:

_____

Principal Investigator

_____

Clint Brown
Director, Office of Sponsored Projects

LAY SUMMARY

We made substantial progress toward completing the goals of Specific Aims 1 and 3 proposed last year. Some unforeseen obstacles hindered progress in Specific Aim 2, which includes development of an assay to screen for drugs that may reverse the functional defects caused by ATP1A3 mutations.

Specific Aim 1 was designed to determine the functional and biochemical consequences of the two most common ATP1A3 mutations found in children with AHC. Our findings lead us to conclude that these two common mutations cause a loss-of-function of the protein coded by ATP1A3 (**Figure 1**). The specific mechanism responsible for the loss-of-function may relate to an inability of the mutant protein to reach the surface of cells (impaired cell surface trafficking; **Figure 2**).

All cells have mechanisms responsible eliminating proteins that have landed in the wrong place. We have demonstrated that lowering the temperature in which the cells are grown can partially overcome these elimination mechanisms and allow the mutant ATP1A3 proteins to reach the cell surface more efficiently (Figure 3). Although lowering body temperature is not a therapeutic option, we will interpret this finding as evidence that the defect in cell surface trafficking can be reversible, ideally by a drug or drug-like molecule.

To complete these studies, we plan to repeat the analysis at least once more to be certain of our findings and to investigate an investigational compound (VRT-325) developed for cystic fibrosis that may also be capable of rescuing mutant ATP1A3 proteins to the cell surface.

Specific Aim 2 proposed to develop a high throughput screening system with which to search for drugs or drug-like molecules capable of restoring normal ATP1A3 function. The basis of this screening system is measurement of fluorescence generated by the interaction between thallium ions and a special dye placed inside cells. Thallium is a surrogate ion for potassium, which is normally transported into cells by the ATP1A3 ion pump. The use of thallium in drug screening experiments is very well established at Vanderbilt.

Progress was made in setting up and testing the thallium screening system using cell lines engineered to express the normal ATP1A3 gene or the two most common AHC mutations. To be successful, the screening system would need to be capable of discriminating between nonspecific ‘background’ fluorescence and specific ‘signal’ fluorescence generated by ATP1A3. Unfortunately, in our initial experiments we did not find an optimal signal-to-background ratio. We will need to continue our efforts to optimize the system before launching the originally proposed drug screening experiments. Several strategies are now being implemented to optimize the system.

Specific Aim 3 proposed to generate induced pluripotent stem (iPS) cells from subjects with AHC. The long term goal of these experiments is to use iPS cells to generate neurons with which we can directly investigate cellular mechanisms of AHC and possibly test candidate pharmacological treatment strategies. Progress has been excellent with the generation of more than 20 different iPS cell lines from AHC subjects with the 3 most common ATP1A3 mutations. We have also gone further to test methods for generating neurons from iPS cell lines. We are now poised to begin performing electrophysiological studies on AHC neurons, something that has never been accomplished before. From these experiments we expect to learn about the fundamental cellular dysfunction that causes AHC, and this may help develop new strategies, or exploit findings from Specific Aim 2, to reverse the underlying defect.

PROGRESS REPORT

We were generously funded by AHCF last year to perform three sets of experiments. The Specific Aims we proposed are:

Specific Aim 1: **To determine the functional consequences of *ATP1A3* mutations associated with AHC.**

Specific Aim 2: **To identify drugs or drug-like small molecules that can correct the functional defects responsible for the disease.**

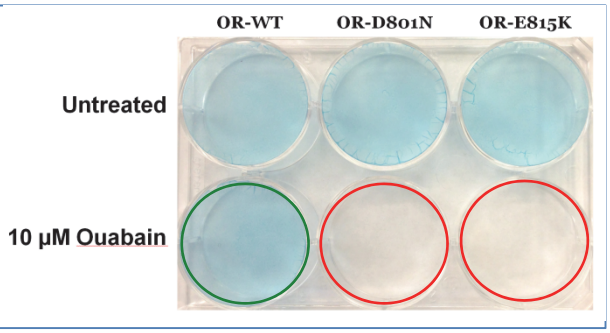
Specific Aim 3: **To establish induced pluripotent stem cell (iPSC) lines from subjects with AHC and ATP1A3 mutations for use as new disease models.**

Substantial progress was made for Specific Aims 1 and 3. The experiments designed to implement a high-throughput assay for drug screening (Specific Aim 2) are ongoing and will be continued into the next year. The following subsections provide a brief summary of research progress.

Specific Aim 1: Functional and biochemical consequences of ATP1A3 D801N and E815K

Initially, to determine if *ATP1A3* mutations conferred either gain- or loss-of-function effects, we employed a cell survival assay that was previously described by researchers investigating the consequences of *ATP1A3* mutations associated with rapid onset dystonia Parkinsonism (RDP). This assay is predicated on the ability of recombinant ouabain-resistant ATP1A3 to protect cultured mammalian cells from ouabain-induced cell death. Cells stably expressing ATP1A3 (wildtype [WT] or mutant) were treated 48 hours with 10 μM ouabain (a potent inhibitor of Na/K-ATPase) and the number of surviving cells were compared to untreated cells. Figure 1 illustrates qualitatively that WT-ATP1A3 expressing cell lines were resistant to ouabain (**green** circle), but cell lines expressing either D801N or E815K (**red**) were highly sensitive. This result indicates that both mutant forms of ATP1A3 encode non-functional ion pumps.

Figure 1 - Cell survival assay indicates loss-of-function for ATP1A3 mutations D801N and E815K. Stable cell lines expressing WT-ATP1A3, D801N or E815K (engineered to have ouabain resistance) were exposed to 10 μM ouabain for 48 hours or left untreated. Cells were stained with methylene blue to visualize. 'OR' indicates ouabain-resistance ATP1A3.



To investigate one plausible cause of loss-of-function (impaired trafficking of mutant ion pumps to the cell membrane), we performed cell surface biotinylation experiments on stable cell lines expressing WT-ATP1A3, D801D and E815K. Figure 2 illustrates our findings that both mutations have significantly lower cell surface expression that WT-ATP1A3, consistent with impaired trafficking to the cell membrane.

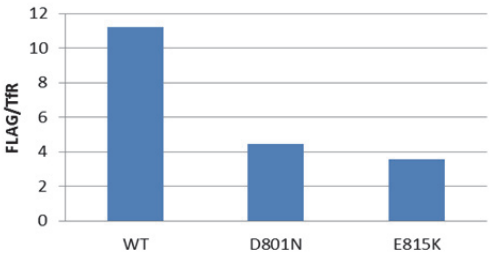
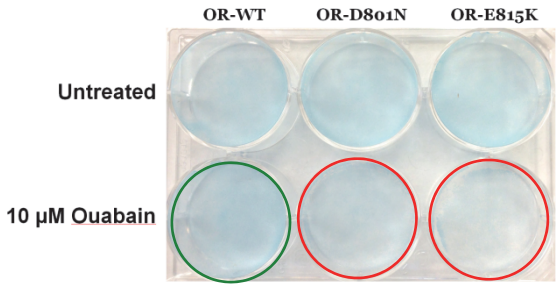


Figure 2 – Cell surface expression of ATP1A3 D801N and E815K is impaired. Summary of results from cell surface biotinylation experiments performed with stable cell lines. Data are normalized to expression of endogenous transferrin.

Interestingly, both mutants retained partial cell surface expression suggesting that the impairment was not complete and raised the possibility that drugs, chemical chaperones or other maneuvers might be capable of rescuing mutant pumps to the cell surface. In a preliminary experiment, we repeated the cell survival assay after cells were incubated overnight at 28°C, a maneuver that has been demonstrated to allow misfolded proteins to escape the cell's quality control mechanisms and promote cell surface expression of mutant membrane proteins. Figure 3 illustrates a partial restoration of cell survival in ouabain-treated cell lines expressing D801N and E815K following overnight incubation at 28°C. This is consistent with partial rescue of impaired trafficking. Quantification of these data is underway.

Figure 3 - Cell survival assay following 28°C incubation indicates partial rescue of loss-of-function ATP1A3 mutations D801N and E815K. (compare with Figure 1). Compare densities of stained cells within **red** circles with that in **Figure 1**.



Specific Aim 2: High throughput assay for drug screening

The goal of this Specific Aim was to optimize a thallium flux assay for use with ATP1A3 for the performance of a high throughput screen of approved drugs and drug-like compounds that can reverse the fundamental defect caused by AHC mutations. The thallium flux method was originally used for studying potassium channels and related ion transporter proteins. The strategy involves loading cells with a thallium-sensitive fluorescent dye, then exposing cells to extracellular thallium ions while continuously monitoring fluorescence. An increase in fluorescent signal from the cells is interpreted as thallium uptake by ATP1A3. An illustration of this assay is shown in **Figure 4**.

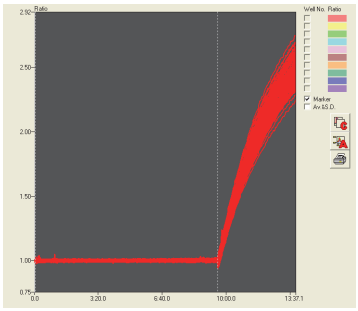


Figure 4 – Thallium-induced fluorescence in HEK-293 cells (red trace) acquired by a Hamamatsu kinetic plate imager. The vertical dotted line shows the time when thallium was added to the cells.

Several experiments designed to optimize detection of thallium-induced fluorescence were completed but the difference between the background signal and the signal evoked by the ATP1A3 transporters was not large enough to proceed with a chemical screen. Two strategies are underway to correct the signal detection problem. First, we are planning to add a second protein subunit of Na/K-ATPase to the cell lines. ATP1A3 encodes the main functional unit of the transporter, but a second subunit (β-subunit) may help boost activity in cells. Although HEK-293 cells, which we are using, are known to have some β-subunit, the quantity may be insufficient to handle the large amount of ATP1A3 we need for the assay. The other strategy involves varying the amount of thallium used in the assay to find the optimal concentration. We are fortunate to have experts at Vanderbilt who established the thallium flux method.

Specific Aim 3: Generation of iPS cells from AHC patients

To date we have generated more than 20 induced pluripotent stem (iPS) cell lines from 5 different patients with AHC who carry *ATP1A3* mutations D801N, E815K and G947R. These lines are in various states of characterization including documentation of proper growth and shape (Figure 4A) and expression of stem cell markers (Figure 4B). We have also differentiated these iPS cells to multiple cell types including mesoderm (muscle) using markers for smooth muscle actin and desmin (Figure 4C). There are active experiments to generate iPS cells from D801N mutation carriers.

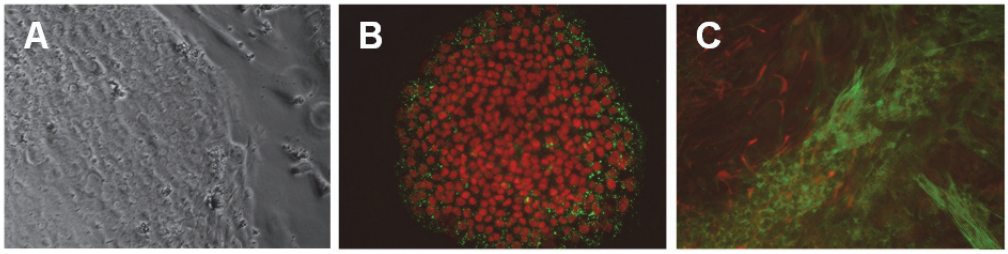


Figure 4 – Induced pluripotent stem (iPS) cells derived from AHC patients. (**A**) Bright field image of iPS cell colony from a patient with ATP1A3 mutation G947R. (**B**) Expression of stem cell markers OCT4 (red) and alkaline phosphatase (green). (**C**) expression of desmin (green) and actin (red) in smooth muscle-like cells derived from iPS cells.

Most recently, we have succeeded in generating neurons from an iPS cell line carrying the mutation G947R (Figure 5) using an embryoid body protocol. This cell line was created from skin fibroblasts from an AHC patient recruited by Dr. Swoboda at the University of Utah.

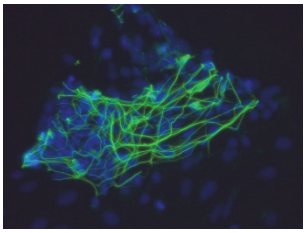


Figure 5 – Neuronal elements from iPS cells having mutation G947R. Green staining is β-tubulin, a marker of neurons. Blue are cell nuclei stained by DAPI.