Molecular Physiology and Pharmacology of *ATP1A3* Mutations in AHC

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Overview and Progress Report – Vanderbilt

Alternating Hemiplegia of Childhood (AHC) is a perplexing neurodevelopmental disease that usually presents at less than 18 months with weakness of one side of the body (hemiplegia) that lasts for hours to days. These events are very frightening to families as well as health care professionals. Incorrect diagnoses are then considered including stroke, brain tumors, and usually epilepsy. Over time, independent alternating involvement of both sides of the body becomes evident, patients can also have episodes of full body weakness. Interestingly, most patients have resolution of their symptoms when they awake from sleep. While most patients are initially misdiagnosed as having seizures or stroke, the pattern of recurrent hemiplegia and recovery in the setting of normal brain MRI scans and lack of any seizure activity eventually leads to a correct diagnosis. This is especially the case as more information and knowledge about AHC is disseminated to the medical community. Later in life, most patients develop developmental delay and intellectual disabilities with variable manifestations ranging from mild to severe.

There is no treatment for AHC that is supported by empirical evidence, most approaches use sleep-inducing agents that can then terminate episodes of hemiplegia. A non-FDA approved drug called flunarizine has been used but there is scant objective evidence supporting its use. While AHC has been recognized as a distinct syndrome for many years, the genetic cause was only discovered in 2013 and is due to mutations *ATP1A3*. This gene encodes a fundamentally important protein pump used by neurons to control the proper levels of sodium and potassium inside cells. While the definition of the genetic underpinnings of AHC was a tremendous advance that gives great excitement to this field of research, we still have only a basic understanding how *ATP1A3* functions, the impact of common and discrete gene mutations, and how new therapies may now be considered based on this information.

Our research has focused on the normal function of *ATP1A3* and how common mutations found in patients with AHC cause neuronal dysfunction. Using a novel functional assay, we recently found that the most common gene mutations that cause AHC impair the flux of sodium and potassium at the surface of the cell. Some of these mutations appear to prevent the protein pump from being correctly “tracked” and suitably expressed on the surface of the cell. This is a tremendous insight as drug discovery approaches may work by simply pushing the abnormal form of the protein to the right locartion in the cell.

An additional and equally exciting avenue of our research has been the creation of human stem cells using skin cells taken from patients with AHC. This technology uses what is called induced pluripotent stem cells (iPSC) and was a huge leap of progress as stem cells can now be made from patient skin cells. This completely avoids any use of embryonic stem cells that remain highly controversial given their origin from human fetuses. iPSC work was so ground-breaking that only 6 years after it was first reported, the inventor of this technology, Dr. Shinya Yamanaka, was awarded the Nobel prize in 2012. The use of patient derived stem cells from patients with AHC then allows us to make human neurons in a dish and directly study the impact of *ATP1A3* gene mutations on neuronal function. Such approaches are becoming more common in biomedical research and are very complementary to more traditional approaches using mice or rats to model human disease. The drawbacks of using iPSC however include their cost of production and maintenance as they require very specialized growth conditions and daily care by skilled research technicians and scientists.

In the Table below, we summarize our iPSC generation to date and also the production of human neurons derived from them. Ongoing efforts have allowed us to produce these cells from all common mutations that cause AHC. Control lines derived from healthy volunteers are also crucial to define normal cellular behavior and *ATP1A3* function. **Figures 1, 2, 3, 4, and 5** below show examples of human AHC patient iPSC, validation of their stem cell status, and derivative neurons.

Patient ID	*ATP1A3* Mutation	iPSC	Neuron
AHC-4 (AHC 35189)	D801N	yes	yes
AHC-8 (AHC 91759)	E815K	yes	yes
AHC-12 (AHC 41935)	G947R	yes	yes
AHC-14 (AHC 46337)	G947R	yes	yes
AHC- 20 (AHC 42103)	G947R	yes	yes
AHC 95454	Splice c.2542+2A→G	yes	In progress
CA	None (control line)	yes	yes
CC	None (control line)	yes	yes
CD	None (control line)	yes	yes

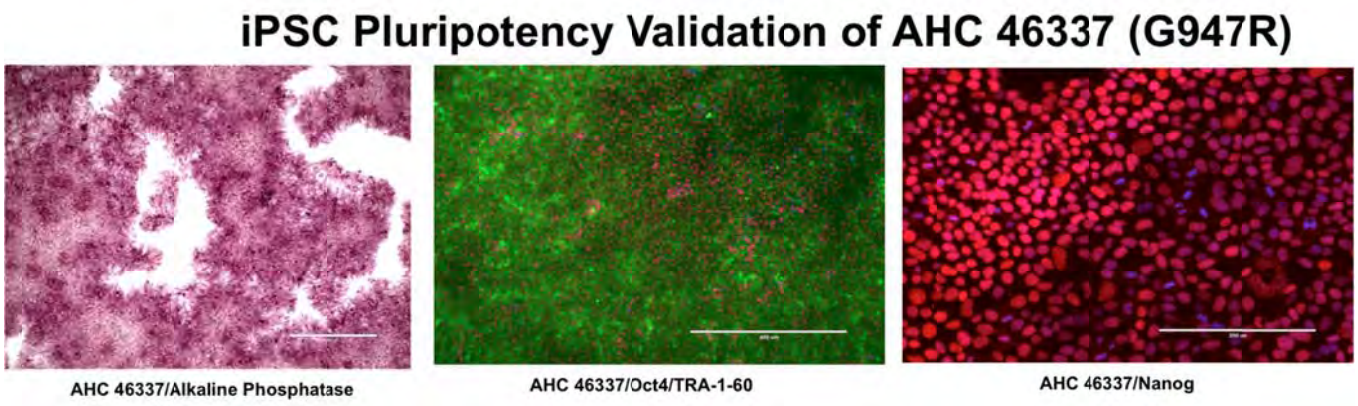

Figure 1. Using a non-integrating episomal method, iPSCs were generated from patient fibroblasts harboring a G947R mutation in the *ATP1A3* gene. Validation experiments included expression of alkaline phosphatase (left image, red stain, size bar 400 microns), Oct4 (red) and TRA-1-60 (green) (middle image, size bar equals 400 microns), and Nanog (right image, red stain, size bar 200 microns).

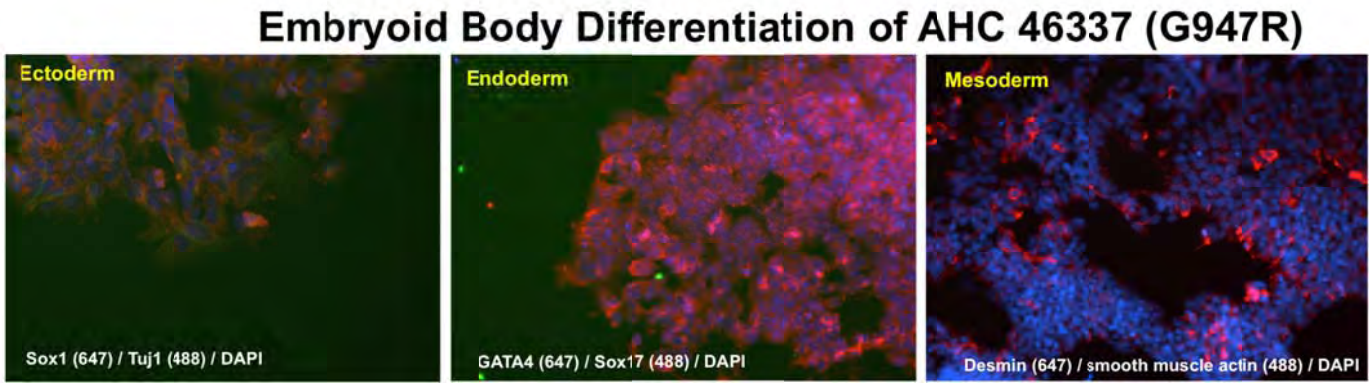

Figure 2. An additional assay of pluripotency was performed to test the ability of iPSCs to generate all germ lines (ectoderm, endoderm, and mesoderm type cells) through embryoid body formation (left image, Sox1 red, Tuj1 (neuronal) green stain); (middle image, GATA4 red stain, Sox17 green stain); (right image, desmin red stain, smooth muscle actin green stain).

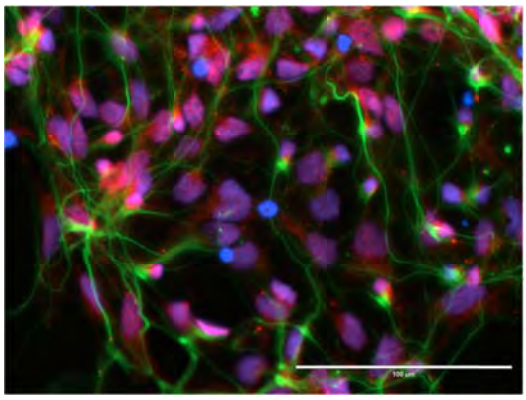

Figure 3. AHC iPSC with G947R mutation were differentiated to neural progenitors (not shown) and then to neurons. Cells were fixed after 98 days in culture. Neurons are positive for Tuj1 (green, neuronal marker), TTF-1 (red, specific neuronal lineage marker), nuclei by Hoechst stain (blue). Size bar equals 100 microns.

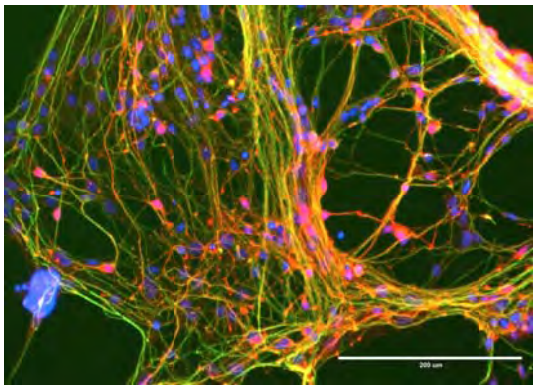

Figure 4. AHC iPSC 42103 with G947R mutation were differentiated to neurons. Neurons are positive for GAD67 (green, inhibitory neuronal marker), PSD-95 (red, post-synaptic neuronal marker), nuclei by Hoechst stain (blue). Size bar equals 200 microns.

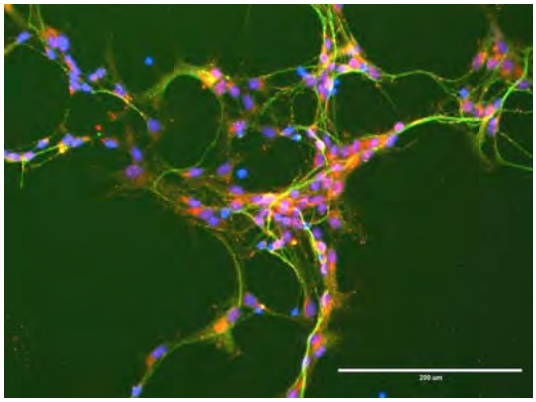

Figure 5. AHC iPSC 42103 with G947R mutation were differentiated to neurons. Neurons are positive for Tuj1 (green, neuronal lineage marker), GABA (red, inhibitory neuronal marker), nuclei by Hoechst stain (blue). Size bar equals 200 microns.

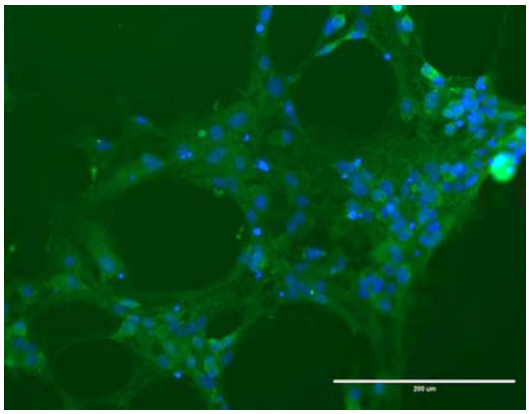

Figure 6. AHC iPSC 42103 with G947R mutation were differentiated to neurons. Neurons are positive for NeuN (green, mature neuronal marker), and nuclei by Hoechst stain (blue). Size bar equals 200 microns.

Progress Report – Northwestern

Screening of FDA-approved drugs that restore mutant ATP1A3 activity

We have continued to use a high-throughput assay system (thallium uptake) to measure ATP1A3 activity in the presence of test compounds (drugs and drug-like chemicals). Although we previously tested a chemical library of all FDA-approved drugs, we had concerns that these results were not optimal. Further, we had not performed a complete screen of the three most common mutations in a systematic manner. Therefore, we purchased a new chemical library of FDA-approved drugs and re-screened each of the three most common AHC-associated ATP1A3 mutations (D801N, E815K, G947R) against each drug in the library. We tested each drug under two conditions: following overnight incubation, and after a short (20 minute) exposure. There were very few drugs that exhibited positive effects on mutant ATP1A3 activity by either exposure paradigm. One compound (chlormadinone acetate), a derivative of the female sex hormone progesterone, showed activity against all three mutations (Fig. 6) with effects on D801N being the most robust. In our prior screen for compounds that rescue E815K, we had also observed an effect of chlormadinone. Therefore, we will be pursing the mechanism for this effect in our future experiments (**Specific Aim 2**).

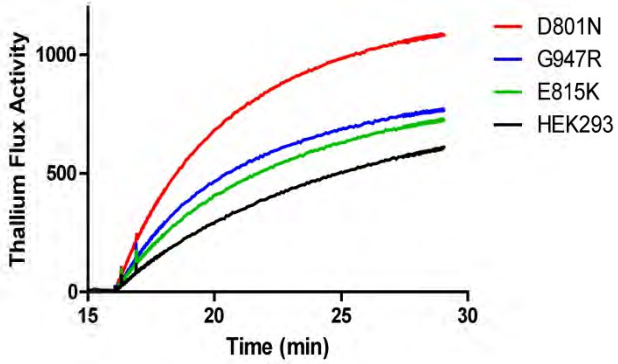

Fig. 6- Activation of mutant ATP1A3 by chlormadinone acetate assayed by thallium flux in HEK-293 cells.

We also screened a second chemical compound library with more than >6,000 entities enriched in structures that are shared by drugs that target ion transporting proteins. This screen used a short exposure paradigm. The results included 71 active compounds that were then assayed again against the ATP1A3 cell line as well as the original HEK-293 cell line (to exclude non-specific effects). Unfortunately, all 71 chemicals activated something in the HEK-293 cells suggesting that none of the effects were specific to ATP1A3. Many of the 71 compounds had a shared chemical structure that might be activating something endogenous to HEK-293 cells. Further assay development efforts are ongoing to explore use of different cell types and to target a more chemically diverse library.

Computational Screening for ATP1A3 Docking Compounds

As an alternative strategy to find small chemical compounds that may correct the functional effects of ATP1A3 mutations, we performed a computer-based screen for compounds that 'dock' to the protein. These studies were performed in collaboration with the Center for Molecular Innovation and Drug Discovery at Northwestern University. The work began by constructing a computer model of ATP1A3 based on previously determined molecular structures (**Fig. 7a**). A potential binding pocket was identified in the vicinity of several known ATP1A3 mutations including D801N (**Fig. 7b**). Next, computer-based docking was performed against a library of 10 million small chemical structures. From this screen, 62 chemical structures were identified that met criteria for docking (examples illustrated in **Fig. 8**). Further testing of these compounds will be performed in **Specific Aim 2**.

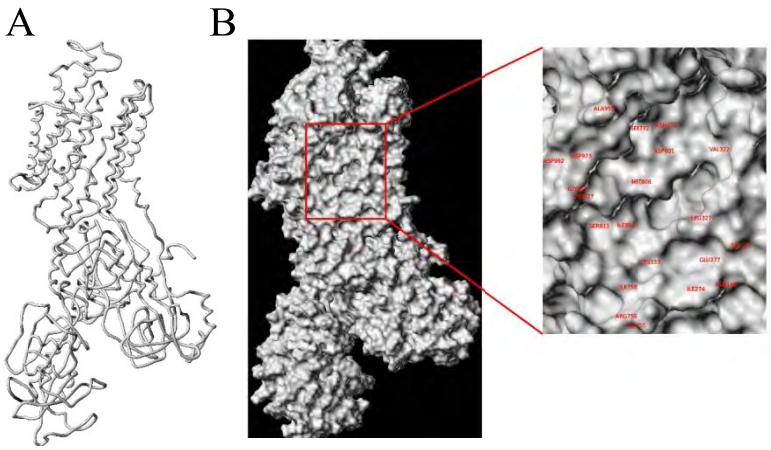

Fig. 7 - Computer modeling of ATP1A3. (A) Structure of human ATP1A3 modeled from the structure of pig kidney Na/K-ATPase α1 subunit. (B) Detailed structure of human ATP1A3 showing binding pocket targeted by chemical screen.

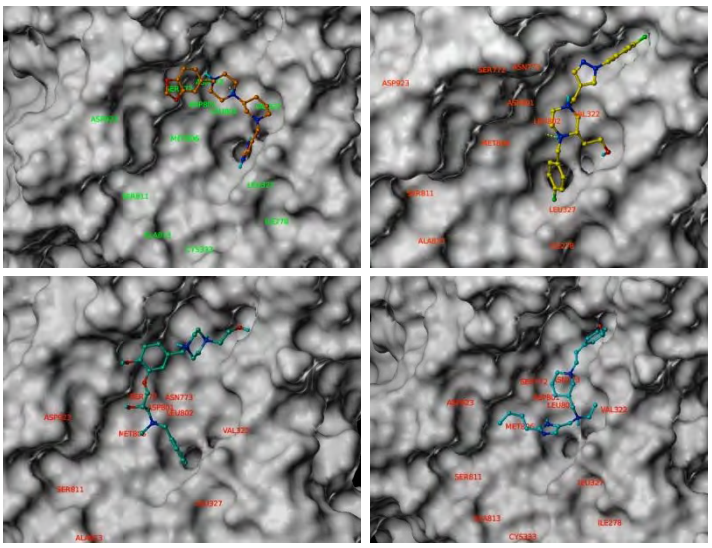

Fig. 8 - Examples of computer-based docking of small chemical compounds to human ATP1A3.