Molecular Physiology and Pharmacology of *ATP1A3* Mutations in AHC

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LAY SUMMARY

Mutations in the gene *ATP1A3* have been identified as the major genetic cause of alternating hemiplegia of childhood (AHC). During the past 2 years, we have made progress in determining the functional and biochemical consequences of the three most common *ATP1A3* mutations (D801N, E815K, G947R) associated with AHC. Our studies have revealed at least two mechanisms for loss-of-function for mutant ATP1A3 transporters. Two mutations (E815K, G947R) exhibit a low proportion of plasma membrane expression implying a defect in delivery of the mutant proteins to the cell surface (e.g., impaired protein trafficking), whereas D801N exhibits low transporter activity but near normal membrane expression. These observations guided us to perform an initial screen of 2,729 drugs and drug-like compounds to find agents capable of restoring transporter activity and plasma membrane expression for E815K and G947R. From this screen, we identified four compounds with activity in a fluorescence-based thallium uptake assay system. In subsequent validation experiments using biochemical techniques (e.g., cell surface biotinylation coupled with western blot analysis), at least two of the compounds show some level of rescue of plasma membrane expression. Additionally, we have succeeded in generating patient-derived induced pluripotent stem cells (iPSC) from subjects carrying the three most common *ATP1A3* mutations (D801N, E815K, G947R) and have successfully generated human neurons from these cells. Collectively, our work has positioned our groups to make additional advances in understanding the molecular and cellular mechanisms responsible for AHC and to exploit this new knowledge for investigating potential therapeutic strategies.

In the next year, we propose to complete the generation and validation of additional iPSC lines representing the most common mutations then further optimize procedures for generating specific types of human neurons from these cells. Next, we will perform comprehensive neurophysiological investigations to determine the cellular abnormalities present in patient-derived iPSC-neurons and use this experimental system to evaluate effectiveness of the compounds previously identified in our screen for restoring normal neuron function. Finally, we will expand our search for compounds capable of restoring ATP1A3 function in engineered cell lines by screening a larger collection (~6,000) of chemicals selected for features known to be active with ion transporting proteins. Additional biochemical studies will be performed to determine if proteasome inhibitors are capable of correcting the apparent trafficking defects exhibited by mutations E815K and G947R. We remain very optimistic that our studies will reveal the molecular and cellular basis for AHC, and provide opportunities to discover potential therapies.

SPECIFIC AIMS

We will build on exciting results obtained from the Ess and George labs during the past year. Our overarching hypothesis is *patients with AHC have mutation specific disease pathophysiology and that neuronal ATP1A3 function can be restored with specific drugs or drug-like compounds*. Through our proposed work, we hope to translate basic science discoveries into new and ultimately more effective therapeutic approaches. The specific aims of our proposal are:

Specific Aim 1 – To generate iPSC-derived neurons from AHC patients.

Specific Aim 2 – To elucidate physiological effects of *ATP1A3* mutations using iPS cell-derived neurons from AHC patients.

Specific Aim 3 – To continue efforts to identify drug or drug-like compounds capable of restoring ATP1A3 function in cells expressing AHC-associated mutations.

PROGRESS REPORT

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

We completed the biochemical assessments of three ATP1A3 mutations (D801N, E815K, G947R). Our data demonstrate convincingly that two of these mutations (E815K, G947R) have severely reduced plasma membrane expression. In other words, these mutations prevent the ATP1A3 protein from reaching the cell surface where it is needed to function (**Fig. 1**). The other mutation (D801N) reaches the cell surface to an extent similar to wildtype ATP1A3 but is not functional for other reasons. We interpreted these findings as evidence that the mutations E815K and G947R, but not D801N, interfere with the normal trafficking of ATP1A3 to the plasma membrane.

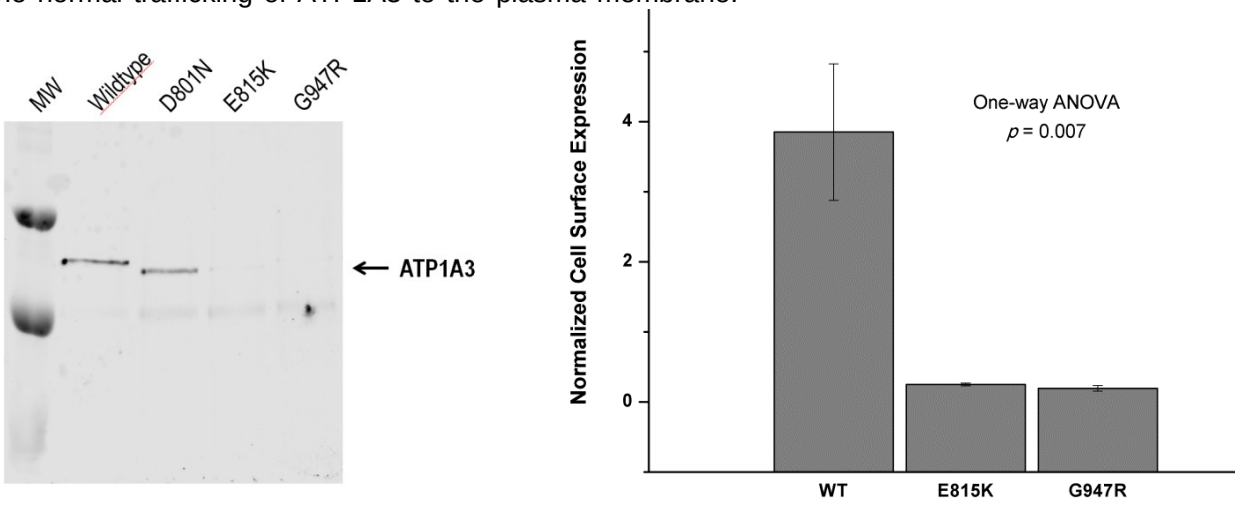


Fig. 1 – Plasma membrane expression of ATP1A3 mutants. ***Left Panel*** - Cell surface biotinylation was performed on HEK cells stably expressing wildtype (WT) ATP1A3 or one of the three most common mutations (D801N, E815K, G947R) associated with AHC. ***Right Panel*** – Quantification of WT, E815K and G947R cell surface expression.

We also optimized and performed a fluorescence-based thallium uptake assay in cells stably expressing ATP1A3 mutants as a platform for performing drug screening and for measuring transporter activity (**Fig. 2**). Results from these studies revealed reduced ion transporter activity for all ATP1A3 mutants. In light of our findings from the biochemical experiments (**Fig. 1**), we concluded that multiple mechanisms are responsible for loss of ATP1A3 function caused by mutations associated with AHC.

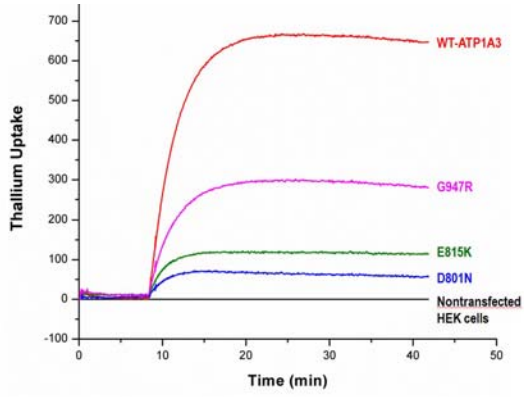
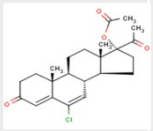
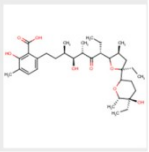
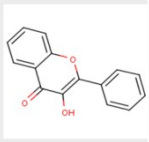
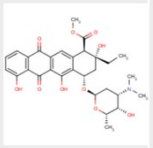


Fig. 2 – Thallium flux assay was performed on HEK-293 cells stably expressing ATP1A3 mutants or HEK-293 cells. Ouabain (10 µM) was added 5 minutes prior to the addition of thallium in order to inhibit the endogenous ATP1A3. Data trace of each cell line represents the average of 8 wells. Thallium flux activity of heterologously expressed ATP1A3 has been normalized by subtracting the activity of HEK-293.

Specific Aim 2: To identify drugs or drug-like small molecules that can rescue the functional and biochemical defects caused by *ATP1A3* mutations.

We designed the assay with results of Specific Aim 1 in mind. Specifically, we have designed the assay to allow us to test compounds for their ability to 'rescue' reduced plasma membrane expression of ATP1A3 mutants and restore functional activity. Mutation E815K gave the strongest 'signal' in our assay optimization trials and therefore we chose this mutation for the pilot screening phase. We screened two collections of drugs and drug-like molecules (NIH Clinical Collections I and II; Spectrum collection) totaling 2,729 compounds. We also tested flunarizine because of this drug's empiric use in AHC patients. All compounds exhibiting activity in the initial screen were subjected to at least two rounds of validation screening against E815K as well as nontransfected HEK cells. There were 37 compounds (1.35%) with activity in the first screen, but only 4 compounds had consistent activity in the validation phase. Flunarizine did not exhibit activity in our tests. The four compounds with validated activity are illustrated in **Table 1**.

Table 1 – Compounds exhibiting validated ATP1A3-E815K cell surface rescue activity measured by thallium uptake assay.

Structure	Formula of Structure	Chemical Name	Therapeutic Use
	C23H29ClO4	CHLORMADINONE ACETATE	progestin, antiandrogen
	C34H54O8	LASALOCID SODIUM	antibacterial
	C15H10O3	3-HYDROXYFLAVONE	
	C30H35NO10	AKLAVINE HYDROCHLORIDE	antibacterial, antineoplastic

We further tested the ability of each compound to rescue plasma membrane expression of E815K and G947R mutations. Lasalocid sodium did not rescue cell surface expression of either mutation. However, chlormadinone acetate and aklavine hydrochloride each boosted plasma membrane expression of E815K consistent with a partial rescue of defective trafficking (**Fig. 3**). Data for 3-hyroxyflavone are being analyzed. Aklavine is a derivative of a natural product (aclacinomycin) that was originally described as a proteasome inhibitor with possible anti-tumor activity. Commercial sources for Aklavine are extremely limited and we are investigating synthesis strategies to enable further studies.

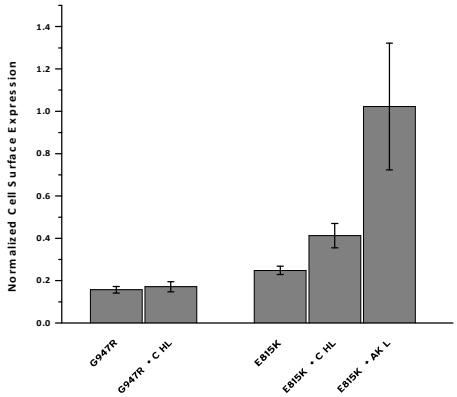


Fig. 3 – Cell surface expression of ATP1A3 mutants in stable cell lines before and after treatment with chlormadinone acetate (+ **CHL**) or aklavine hydrochloride (+ **AKL**).

Specific Aim 3: To elucidate electrophysiological effects of *ATP1A3* mutations using iPS cell-derived neurons from AHC patients.

We successfully generated induced pluripotent stem cells (iPSC) using fibroblasts obtained from multiple patients with AHC. These included fibroblasts obtained by Dr. Kathy Swoboda (University of Utah) as well as patient samples obtained by Dr. Ess through the Vanderbilt AHC Clinic. All patients enrolled in the study underwent a thorough clinical phenotyping by AHC experts. Each patient fibroblast line was used to generate multiple independent lines of iPSC using non-integrating plasmid transfection technology (Okita et al., 2011). This is essential to generate high quality iPSC. In contrast, the use of retroviruses by many groups compromises stem cell quality. Furthermore, potential functional assays may be negatively impacted as retroviruses are permanently integrated into the genome of target cells. The viral genes can then be reactivated and affect cellular identify, particularly during neuronal differentiation. The three most common ATP1A3 mutations (**Table 2**) are represented among the iPSC lines we have made.

Table 2 – Induced pluripotent stem cell (iPSC) lines generated from AHC patients.

Patient ID	*ATP1A3* Mutation	Notes
AHC-2	D801N	Same mutation but different patient than below, developmental delay, possible epilepsy
AHC-4	D801N	In AHCF database, obtained from K. Swoboda
AHC-8	E815K	In AHCF database, obtained from K. Swoboda
AHC-12	G947R	Same mutation but different patient than below, developmental delay, possible epilepsy
AHC-14	G947R	In AHCF database, obtained from K. Swoboda
CA	none	Control lines
CC	none	Control lines
CD	none	Control lines

Concurrent with generation of iPSC, we have made excellent progress in directed differentiation of stem cells to desired neuronal cell types. This is a complicated process that involves growth of iPSC on specialized substrates with addition of pro-neuronal growth factors and removal of stem cell maintenance media. Using these protocols, we can recapitulate the developmental processes used during normal human brain development with differentiation of iPSC first to neural progenitor cells (by definition restricted in their differentiation potential to neuronal and glia cells) and then to produce of specific subclasses of neurons. While many types of neurons can be generated, we focused on the production of neurons that use glutamate or GABA as neurotransmitters. These cells (glutamatergic or GABAergic) are the main excitatory and inhibitory neurons respectively of the human cortex. These neuronal sub-types were selected as the intriguing clinical phenotypes seen in patients with AHC could be hypothesized to be due to either excess excitation or loss of inhibition. The resulting imbalance of excitatory or inhibitory inputs would destabilize any neural network and could certainly be expected to underlie neurologic symptoms such as episodic weakness, seizures and developmental delay.

We have devoted many resources to optimize our neuronal differentiation protocols can now routinely generate neural progenitor cells at greater than 80% efficiency (Neely et al., 2012) (**Fig. 4**). We have also worked to optimize the production of specific neuron subclasses although the overall efficiency is lower (**Fig. 5**). Differentiating iPSC to GABAergic neurons is an active area of research in the Ess laboratory given the focus on phenotypes of epilepsy, developmental delay and autism. All of these important neurologic disorders are thought to be due to dysfunctional inhibitory neurons.

Finally, we have developed strategies (**Fig. 6**) to identify specific types of neurons within live cell cultures. This will permit us to use flow cytometry as an approach to collect enriched batches of specific

cell populations for use in functional neurophysiological studies in collaboration with the George laboratory. In preparation for implementing our planned long distance collaboration with Dr. George's laboratory, we have development a procedure to freeze neurons and ship them to Chicago where members of the George lab will thaw, grow and study the cells.

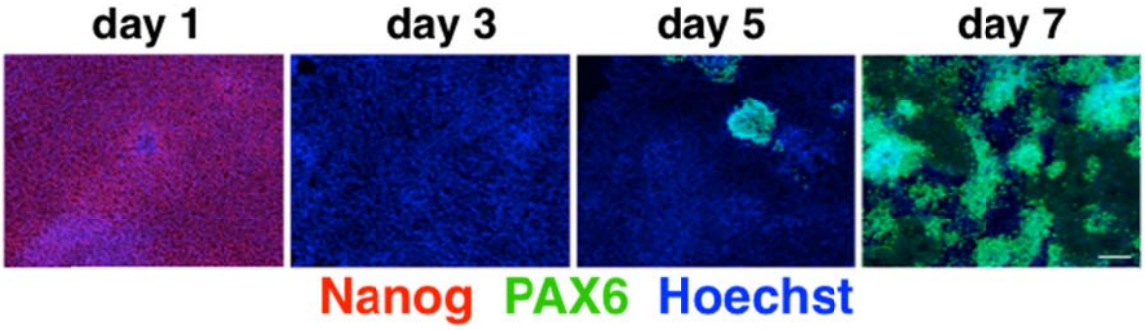


Fig. 4 - Efficient Differentiation of human iPSC (Nanog^{+}) to human neural progenitor cells (Pax6^{+}) in 7 days using a modified BMP inhibition protocol. Hoechst signal indicates cell nuclei.

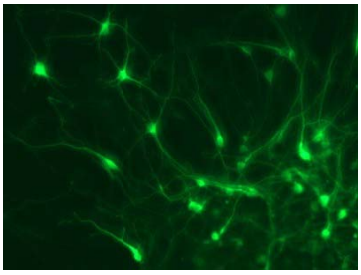


Fig. 5 - GABAergic neurons generated from iPSC line with a D801N mutation in the *ATP1A3* gene, patient AHC-4. Green fluorescence indicates anti-GABA antibody binding. Cells were differentiated for 60 days before fixation and immunostaining.

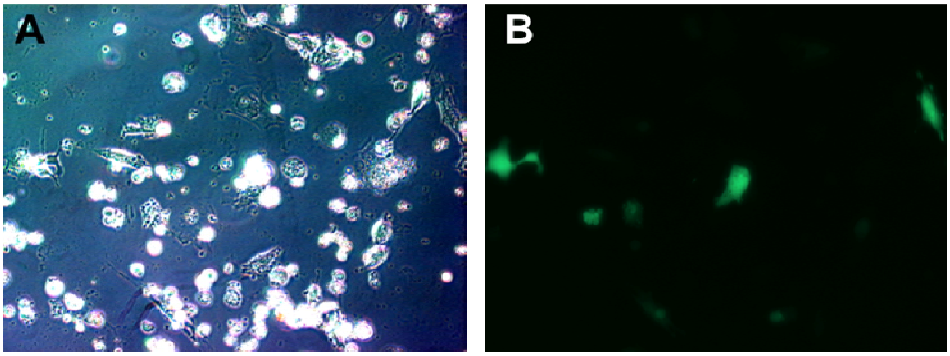


Fig. 6 - Identification of GABAergic neurons using adenovirus labeling of live neurons harboring the D801N mutation in the *ATP1A3* gene, derived from patient AHC-4. **A**. Brightfield image of live neuronal culture derived from patient AHC-4 iPSC lines. After 60 days of neuronal differentiation, cells were transduced with an adenovirus expressing GFP under the control of the GABAergic neuronal specific promoter GAD67. Fluorescent green cells seen in **B** represent a subset of neurons that are readily observable in live cultures. This allows selective electrophysiology experiments to determine the functional consequences of the D801N mutation and other mutations in *ATP1A3* and to assess the impact of potential therapeutic compounds.