Molecular Physiology and Pharmacology of *ATP1A3* Mutations in AHC

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Progress Report – Vanderbilt

Final Characterization of induced Pluripotent Stem Cell Lines

Over the last year, we completed our generation and characterization of iPSCs from patients with the three most common mutations (D801N, G947R, and E815K) in *ATP1A3* gene. These assays include 1) validating expression of pluripotent makers such as Nanog, alkaline phosphatase, and OCT4, 2) ability to form embryoid bodies and generate ectoderm, mesoderm, and endoderm derivative tissues, 3) confirm normal numbers of chromosomes, and 4) non-genomic integration of the DNA plasmids used for reprogramming. These assays have been done in multiple lines from patients with the three most common *ATP1A3* mutations. We now have characterized and frozen at least 2 independent lines (**Figure 1**) with D801N, G947R, and E815K mutations. In addition, these lines have been shipped to the George lab in Northwestern. This is for their ongoing experiments (see below) but also to ensure that this precious resource is safely distributed as a back up against any catastrophe that may affect our stem cell facility. This redundant sharing will be the basis for future distribution of these stem cell lines with other scientists throughput the world interested in pursuing AHC and related research.

Cell Line	Pluripotency Markers				Embryoid Body		Integration
	AlkPhos	TRA-1-60; Oct 4; SSEA4	SSEA3	Nanog	Desmin; SMA; Sox1	β-tubulin 3 GATA4; Sox17	Integrated?
AHC 95454-3	yes	yes	yes	yes	yes	yes	no
AHC 42103-51	yes	yes	yes	yes	yes	yes	no
AHC 42103-52-B	yes	yes	N/A	N/A	yes	yes	no
AHC 95454-1	yes	yes	yes	yes	yes	yes	no
AHC 46337-72	N/A	yes	yes	yes	N/A	N/A	no
AHC 91759-24	yes	yes	yes	yes	N/A	N/A	no
AHC 35189-6	yes	yes	yes	yes	N/A	N/A	no

Fig. 1. Summary of iPSC line characterization for AHC patient lines 95454 (Splice recognition mutation), 42013 (G947R), 46337 (G947R), 91759 (E815K), and 35189 (D801N).

Neuronal Differentiation

We have continued our neuronal differentiation protocols and have considerably refined them over the past year. While our goal is to make different and distinct neuronal lineages (those using glutamate, GABA, acetylcholine, or dopamine as their main neurotransmitter), we have hypothesized that much of the neurologic signs and symptoms seen in AHC is due to GABAergic neuronal dysfunction. We have then focused our efforts on the efficient and specific generation of these neurons. As shown in **Figures 2** and **3**, we have increased our yield though these are still lengthy protocols, taking up to 90 days. Given this length of time and our future

need for higher throughput experiments, we have investigated the impact of freezing lines at various stages of differentiation. This approach will also very focused experiments to be initiated at a partial stage of differentiation and repeated as necessary to ensure results and reproducible and reliable.

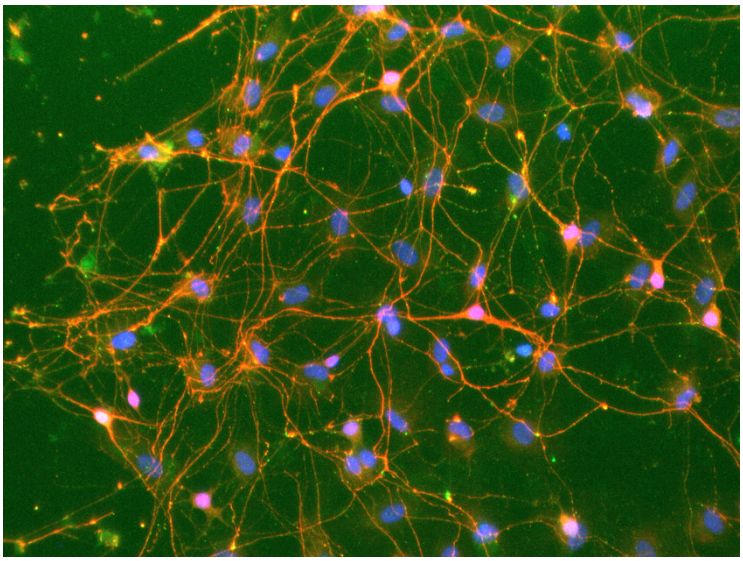

Fig. 2 GABA (lineage marker, green) and NeuN (mature neuronal marker, red) from differentiated neurons derived from AHC patient stem cells lines. Punctate green expression likely indicates inhibitory synapses. Magnification 20X.

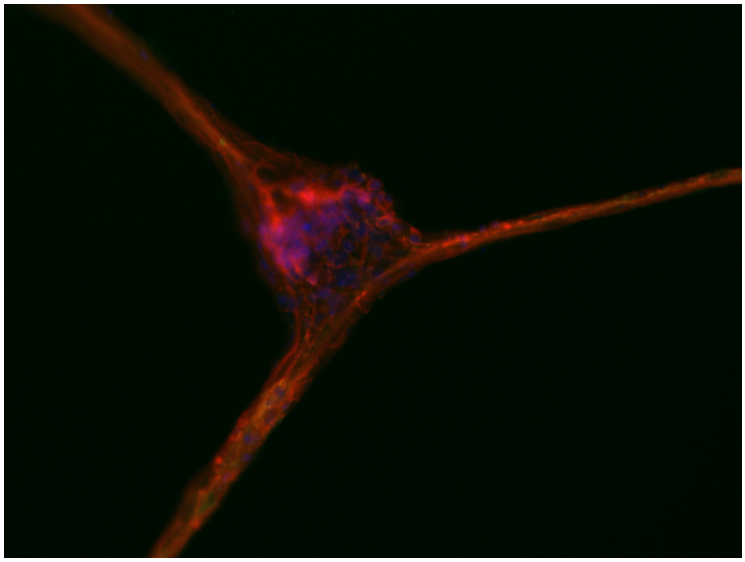

Fig. 3 ATP1A3 and MAP2 expression in neuron obtained from stem cells with the G947R mutation (AHC subject 42013). After differentiating for 133 days, cells were fixed and immunostained for ATP1A3 (green) and MAP2 (red, mature neuronal marker). Magnification 40X.

Na/K ATPase Functional Assays in Human Neurons with ATP1A3 Mutations

The thallium flux assay has been optimized by the George lab to test the function of the Na/K ATPase in living cells. This has been used to screen compounds for the ability to rescue cells with AHC causing mutations in the *ATP1A3* gene. We have modified this approach using human neurons differentiated as above as shown in **Figures 2** and **3**. The rationale for this is to study the most relevant cell type and to further address the question of neuronal lineages in AHC. That is, what is the impact of *ATP1A3* mutations on GABAergic versus other lineages in terms of Na/K ATPase function? These inquiries will greatly help focus future efforts as well as the design and interpretation of mouse models. This will help ensure we have the best tools for studying ATP1A3 function that can then most rapidly be translated to human therapeutic trials.

The use of iPSC derived human neurons will be employed to further validate the efficacy of any identified compounds (see below) can also reverse cell dysfunction.
With this rationale in mind, we differentiated control and AHC patient (42103, G947R) derived stem cells to neurons. We then directly tested the ability of the endogenous Na/K ATPase pump compared to neurons derived from healthy volunteers. Our very exciting results are shown in **Figure 4**, G947R mutant human neurons clearly have impaired pump function. This is very similar to results we have previously shown using genetically engineered HEK cells that express either the wild type or mutant alleles of *ATP1A3*. The important distinction here is that we are now able to use intact human neurons to test pump function and to discover/validate compounds and drugs that van be used to reverse pump abnormalities. HEK cell lines will still be used for bulk screening experiments but the use of human cells is critical as ALL OTHER required proteins and co-factors for pump function are present in human neurons but may not be present in HEK cells. Furthermore, the use of neurons derived from specific patients with AHC opens the door to individualized studies. This will allow us to start to address variability in AHC, why do some patients have relatively mild, moderate, or severe courses? Are there other modifier genes that control these outcomes? With such information, can we design therapeutic approaches and change the course of the disease?

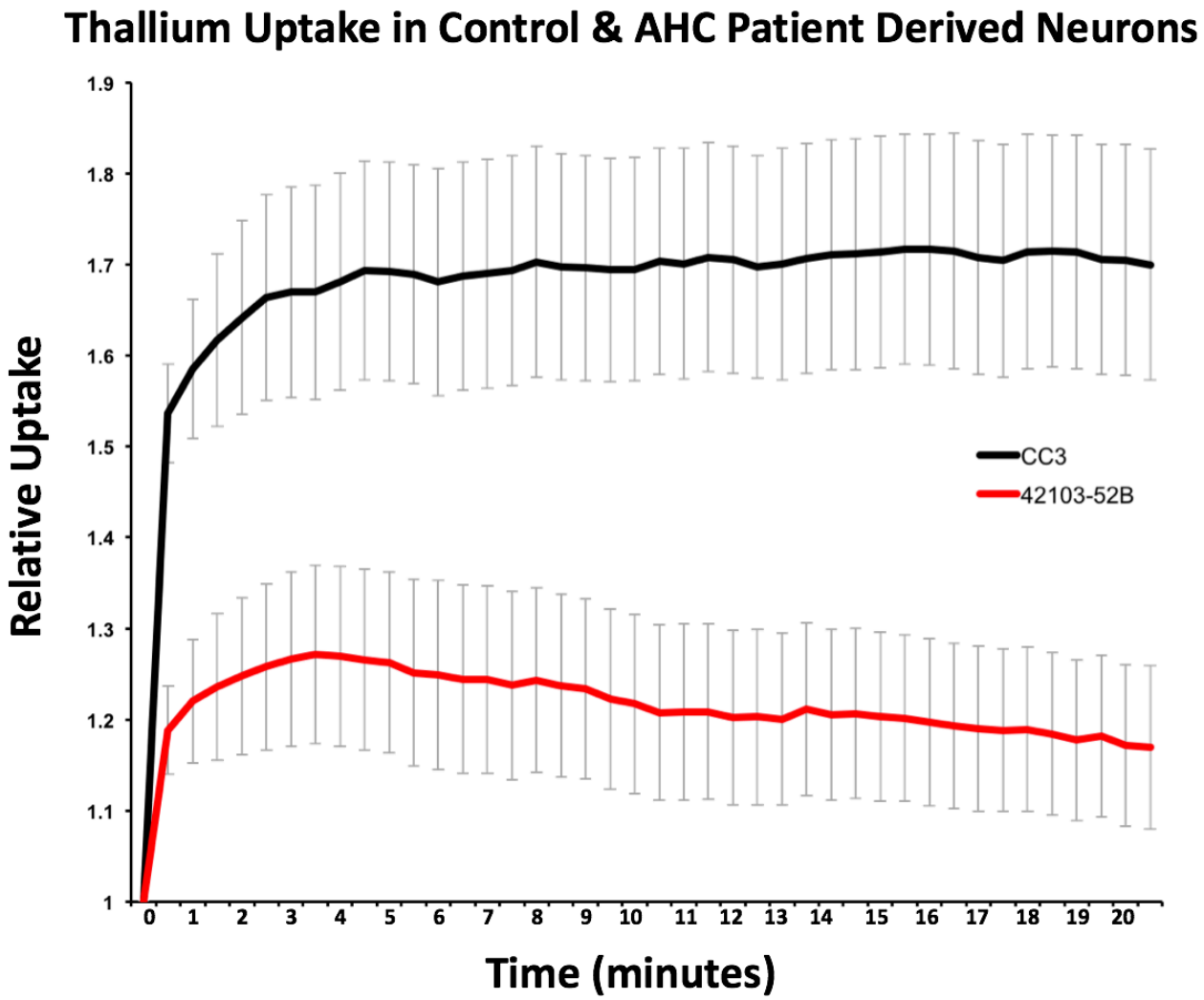

Fig. 4. Control (CC3) and AHC patient derived (42103-52B) neurons were incubated with

preloaded with a thallium responsive fluorescent dye, then incubated with thallium. Images were acquired every 30 seconds from 100s of neurons. The relative fluorescent signal was markedly reduced in human neurons (predominantly GABA expressing) compared to human neurons derived from a healthy control subject.

Progress Report – Northwestern

Computational Screening for ATP1A3 Docking Compounds

Last year, we initiated collaboration with the Center for Molecular Innovation and Drug Discovery at Northwestern University to use computer-based drug screening to find chemical compounds that would bind to and potentially rescue abnormal ATP1A3 function caused by AHC mutations. A screen of ~10 million small chemical structures was then performed and this resulted in the identification of 55 compounds. During the past several months, we acquired these compounds and performed cell-based assays to determine if any were able to rescue the effects of AHC mutations.

We devised a modified cell-survival assay that can be performed in 96-well plates and would allow for quantitative analyses of the results. In this assay, cells that stably express a ouabain-resistant form of ATP1A3 (wild-type or mutant) are tested for their ability to survive in the presence of ouabain. Cells that do not express ouabain-resistant ATP1A3 or nonfunctional mutations do not survive after 48-hour exposure to 30 μM ouabain. However, if a compound were able to rescue the function of mutant ATP1A3, then the cells would survive. Using this cell-survival assay strategy, we demonstrated that 5 of the compounds identified by the computer-based screen could partially rescue the effect of ATP1A3 mutation D801N (**Figure 5**) without having a nonspecific effect on the background cell line (HEK). By contrast, we saw less survival in cells expressing the ATP1A3 mutation D801Y, which differs very slightly from D801N. We are now completing additional experiments to demonstrate the effect across a range of compound concentrations (dose-response relationship) as further evidence that the observed effects are specific to rescuing ATP1A3. We are also planning to test effects of these compounds on cells expressing mutation G947R.

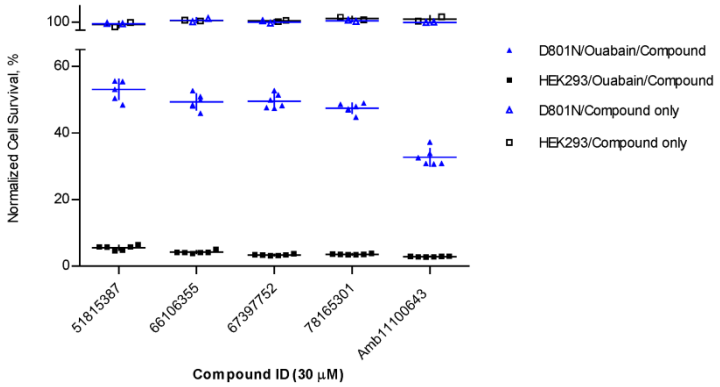

Fig. 5 - Cell survival assay results for 5 compounds identified by computer-based screening. These compounds rescued cell survival from ~30% (#Amb11100643) to ~55% (#51815387).

Computer docking experiments showed how these compounds fit snuggly within a binding pocket neighboring the amino acid position affected by these two mutations. More specifically, we observed that the mutant residue (asparagine) actually makes hydrogen-bonds with the

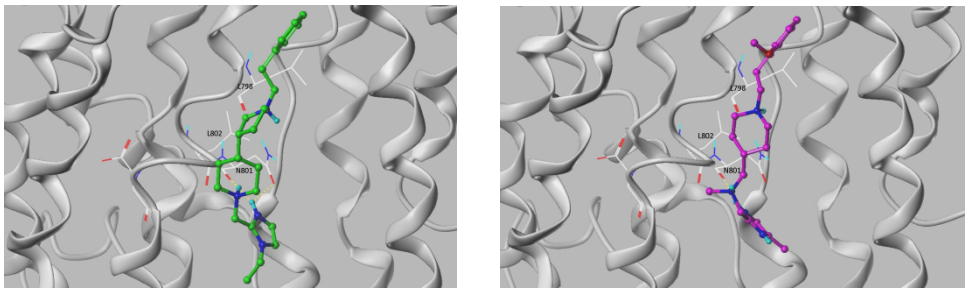

Fig. 6 - Representative images of compound docking to ATP1A3-D801N. Compounds illustrated have ID 51815387 and ID 66106355. Dotted yellow lines indicate hydrogen bonding between the mutant asparagine and the compounds.

compounds (**Figure 6**). We are now examining how the other mutant residue (tyrosine in D801Y) changes this binding pocket. This information will be valuable for identifying or designing other compounds that fit more tightly in this pocket with the goal of finding compounds that more fully rescue the loss-of-function effects of ATP1A3 mutations.

Electrophysiological studies of AHC neurons

Since last fall, we have made great progress in growing neurons from the induced pluripotent stem cell (iPSC) lines generated from AHC patients. Two classes of neurons have been grown most successfully and these have offered the first opportunities to investigate their electrophysiological properties. We were first successful generating neurons that resemble spinal motor neurons, and later have been successful generating cortical excitatory neurons (**Figure 7**). Our original attempts to culture previously frozen cortical inhibitory neurons generated in Dr. Ess' lab at Vanderbilt have not been successful. However, we believe this is due to the need for additional cell types – glial cells – in the cultures. Ongoing experiments will test this idea.

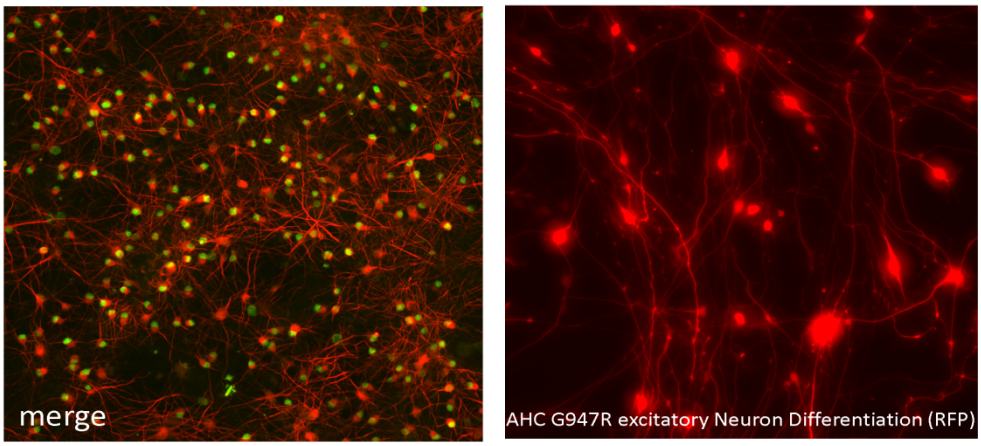

Fig. 7 - iPSC-derived neurons. Left panel - motor neurons stained with a neuronal marker (MAP2; red) and a marker specific for motor neurons (Islet 1, green). Right panel illustrates cortical excitatory neurons (red) from a patient with AHC mutation ATP1A3-G947R.

Because we were unsure of the relevance of iPSC-derived motor neurons to AHC, we examined these neurons for the expression of ATP1A3. Data from previous gene expression studies performed on these neurons by Dr. Evangelos Kiskinis (colleague at Northwestern) were examined and we found clear evidence of expression of ATP1A3 RNA in the cells. We also performed immunohistochemical analyses with ATP1A3-specific antibodies and demonstrated clear evidence for ATP1A3 expression in the neurons (**Figure 8**). Further review of the literature revealed previous studies reporting robust ATP1A3 expression in spinal cord neurons in rodents. Hence, we believe that motor neurons can be an appropriate cell model with which to investigate basic neurophysiological properties of AHC neurons.

Using iPSC-derived motor neurons, we have succeeded in recording neuronal action potentials from cells derived from an AHC patient carrying the G947R mutation. For these experiments, we examined motor neurons generated from the AHC iPSC line and compared activity to similar neurons from a control (non-AHC) cell line. The results were rather remarkable in that neurons from the control iPSC line exhibit robust action potential firing whereas neurons form the AHC iPSC line had essentially no activity (**Figure 9**). This experimental result demonstrated feasibility

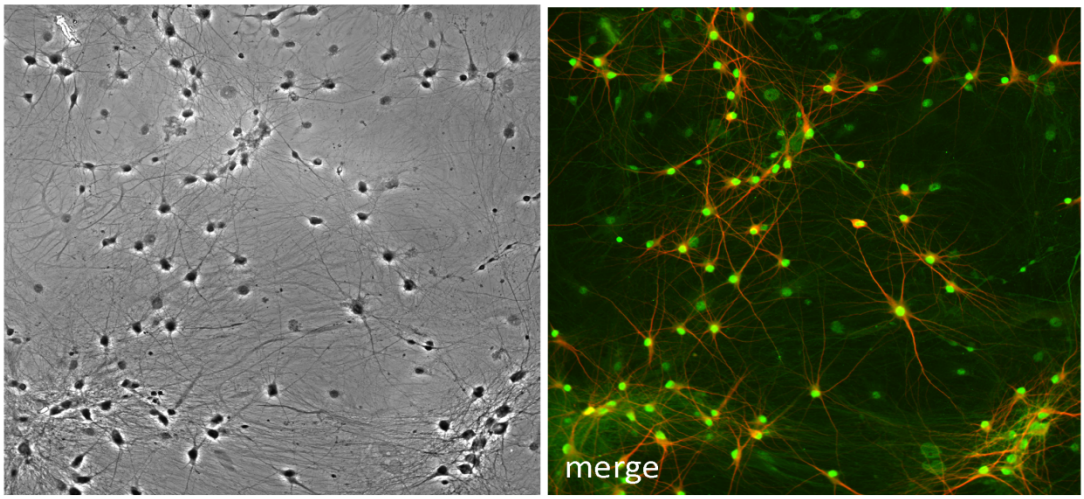

Fig. 8 - Expression of ATP1A3 in human iPSC-derived motor neurons. Left panel illustrates neurons on a lawn of glial cells. Right panel shows green fluorescence indicating ATP1A3, and red/orange indicating overlap between ATP1A3 and the neuron marker MAP2.

of recording from human AHC motor neurons and suggested there may be dramatic differences in neuronal excitability underlying this condition. We are actively working to repeat these experiments on separate batches of neurons.

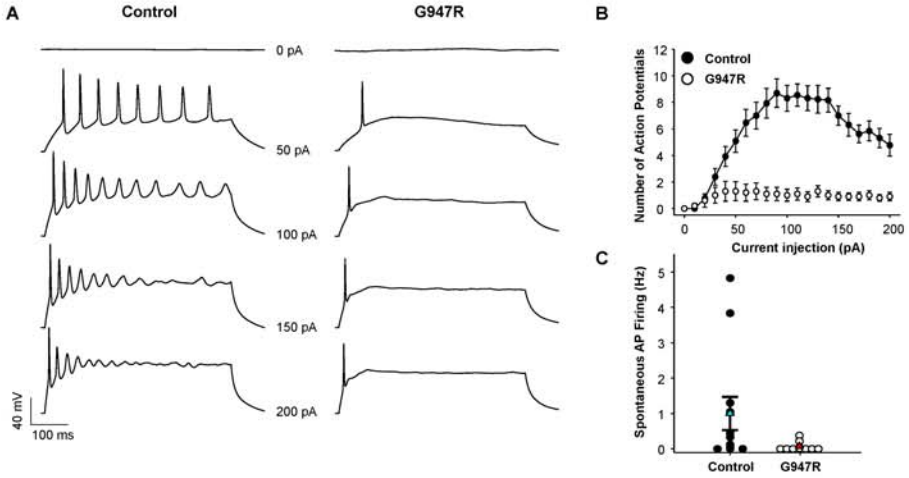

Fig. 9 - Electrophysiological recordings from iPSC-derived motor neurons from a healthy person ('control') and a person with AHC caused by ATP1A3 mutation G947R. (A) Evoked action potentials. (B) Correlation of number of action potentials evoked at each level of injected current. (C) Number of spontaneous action potentials in control vs G947R neurons.

Generation of a mouse model of AHC caused by ATP1A3 mutation G947R

We have introduced the *ATP1A3* mutation G947R into the mouse genome using a state-of-the-art molecular editing approach employing the bacteria CRISPR/Cas9 system. Using this approach, we designed molecular reagents capable of targeting the region of the ATP1A3 gene where the G947R mutation occurs. We initially tested this system in a cultured mouse cell line (3T3 cells) and demonstrated successful targeting. The next step designed to replace the native sequence of the gene with the G947R mutation proved much more challenging because of the nature of the surrounding sequence. However, we eventually succeeded in introducing the mutation in 3T3 cells with high fidelity. We then enlisted the services of the Northwestern transgenic mouse core facility to introduce the mutation in mice. Mouse blastocysts were injected in mid-April with the targeting reagents and we are now waiting for the first offspring to be born. As a backup, we are planning a second round of injections in early May. If all goes according to plan, then we expect to have G947R positive mice in May 2016.