Award Number: W81XWH-12-1-0013

TITLE: Whole Exome Analysis of Early Onset Alzheimer's Disease

PRINCIPAL INVESTIGATOR: Margaret Pericak-Vance

CONTRACTING ORGANIZATION: University of Miami
Miami, FL 33136

REPORT DATE: April 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE		Form Approved OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2017	Annual	1March2016 - 31March2017

4. TITLE AND SUBTITLE
Whole Exome Analysis of Early Onset Alzheimer's Disease

5a. CONTRACT NUMBER
W81XWH-12-1-0013

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Margaret A. Pericak-Vance, Ph.D.

E-Mail: mpericak@med.miami.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Miami, 1400 NW 10th Avenue
Miami, FL 33136

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The primary focus toward identification of Alzheimer disease (AD) risk genes over the past five years has been testing the common disease common variant (CDCV) hypothesis through the use of genome-wide association studies (GWAS) in late onset Alzheimer disease (LOAD). While common variation clearly plays a role in AD there is a growing realization that the CDCV hypothesis is unlikely to explain all the genetic effect underlying AD. One alternative hypothesis invokes multiple rare variants (RV) in one or more genes, each with stronger individual effects than CDCV genes. We designed this project to test the rare variant hypothesis in AD by examining those cases with the most severe phenotype as determine by early onset (EOAD, cases with AAO < 60 years). Although there are three known EOAD genes (PS1, PS2 and APP) they account for only ~60-70% of familial EOAD and even less of sporadic EOAD. Thus, the majority of the genetics of EOAD remains unknown. Until now, large extended families with AD in multiple generations were necessary to identify variants of significant effect contributing to AD risk, however, with the advent of new genomic technologies such as high-throughput sequencing technology, small family aggregates and isolated cases, particularly those with an extreme phenotype of the disorder (such as early onset) can be used. Thus, we will utilize whole exome high-throughput sequencing to identify high risk AD variants that we will further characterize with respect to AD. We will examine both Caucasian and Caribbean Hispanic AD populations. Our two pronged approach includes structural characterization at the DNA level (Dr. Pericak-Vance), and analysis of Caribbean Hispanics (Dr. Richard Mayeux). Comparing across populations will be extremely useful. Specifically, high priority RVs identified through the whole exome analysis will be further explored with multiple strategies. We will also genotype the interesting variants in a large sample of late-onset (LOAD) cases to examine their involvement in all AD. We will thus prepare a list of high priority candidates for additional follow-up and functional analysis.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	26	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

INTRODUCTION:

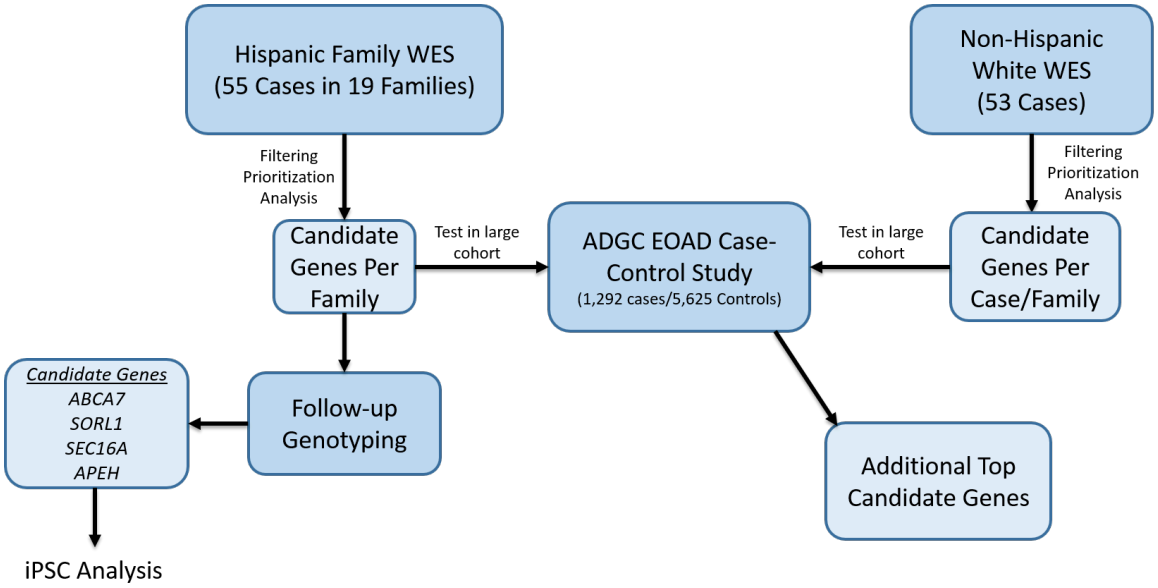
The primary focus in the identification of Alzheimer disease (AD) risk genes has focused on the common disease common variant (CDCV) hypothesis using genome-wide association studies (GWAS) in late onset Alzheimer disease (LOAD). It is clear that common variants play an important role in AD, the CDCV hypothesis can't fully explain the genetic factors underlying AD. As an alternative, recent genetic studies have focused on the identification of multiple rare variants (RV) in one or more genes, each with string effect sizes. To that end, the current study was designed to test the rare variant hypothesis in AD by examining those cases with the most severe phenotype as determine by early onset (EOAD, cases with AAO < 60 years). There are three known EOAD genes – *Presenilin 1* (*PS1*), *Presenilin 2* (*PS2*), and *Amyloid precursor protein* (*APP*) – that account for ~60- 70% of familial EOAD cases and fewer in sporadic EOAD and, as such, the majority of EOAD genes remain to be identified. To that end, we will utilize whole exome next generation sequencing (NGS) to identify high risk AD genetics variants. We will examine both Caucasian and Caribbean Hispanic AD populations. Our two pronged approach includes structural characterization at the DNA level (Dr. Pericak-Vance), and analysis of Caribbean Hispanics (Dr. Richard Mayeux). Comparing across populations will be extremely useful. Specifically, high priority RVs identified through the whole exome analysis will be further analysis, including bioinformatics and computational analysis, genotyping of variants in a large sample of late-onset (LOAD), as well as, functional characterization using patient-specific induced pluripotent stem cells (iPSCs). Patient specific iPSC derived from EOAD patient samples bearing genetic variants of interest will be developed and differentiated into forebrain neurons that will be characterized for markers of AD pathogenesis, including expression of pathogenic amyloid beta and tau isoforms.

BODY:

ACCOMPLISHMENTS:

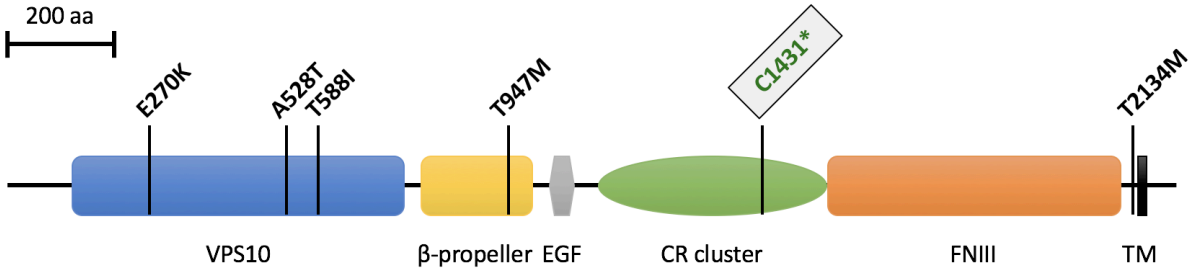
Phase I – Sequencing and Validation of Variants of Interest

We have completed analysis of a comparison between the Alzheimer's Disease Genetics Consortium (ADGC) early onset Alzheimer's disease exome chip case-control association study and the WES produced from this project. Nine genes are genome-wide significant at a Bonferonni correction for 7,249 genes tested, including *PSD2* ($P=6.98 \times 10^{-7}$), an endocytic gene with 2 rare, missense variants present in two separate NHW EOAD cases. Below is the flow chart of the study, with the most interesting genes and variants being funneled into the iPSC analysis.



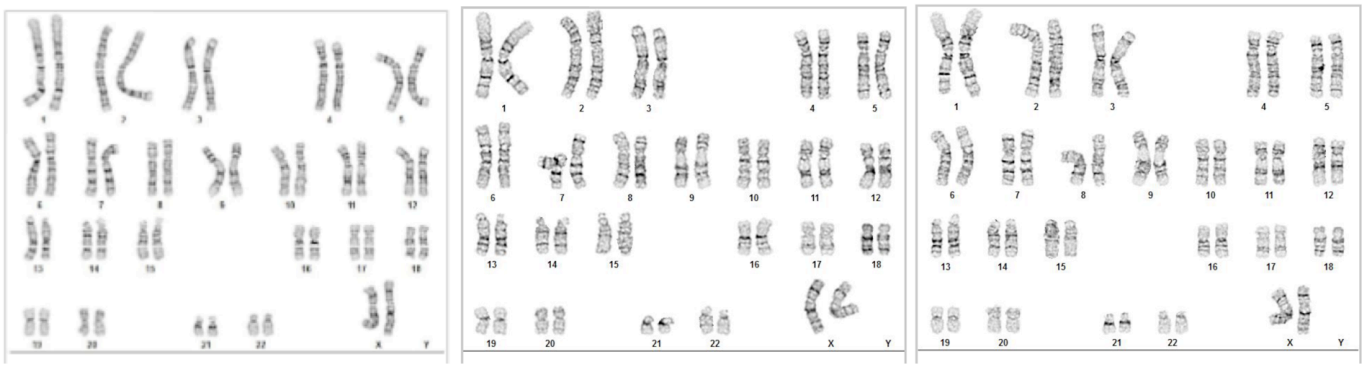
Phase II – AD iPSC Functional Studies

For the generation of iPSC lines, we have collected PBMCs from EOAD non-Hispanic white individuals bearing an alteration in the *SORL1* (shown below), African American individuals carrying an ethnic-specific deletion in the *ABCA7* gene, and Caribbean-Hispanic (Dominican Republic) individuals bearing variants in the *SEC16A* gene. Two iPSC lines has been made from individuals with the *SORL1* from two siblings, one with AD and another with mild cognitive impairment, often a precursor to AD. In addition, iPSC lines have ben generated from an AD patient another with the *SEC16A* alteration, and two patients carrying with the *ABCA7* deletion.

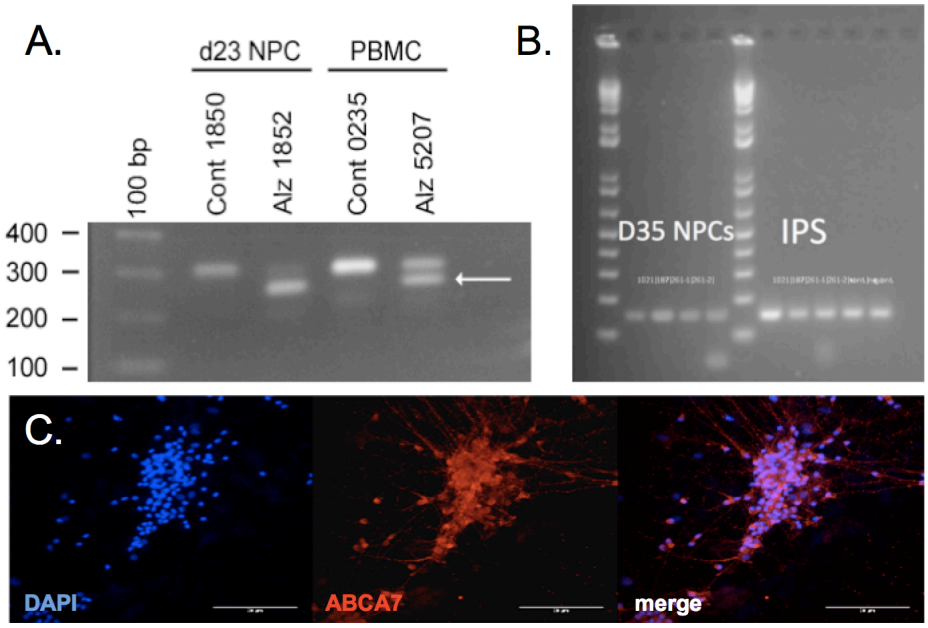


The frameshift alteration in the SORL1 protein falls in the CR-cluster, which is essential for interaction with APP. Removing the CR-cluster abolishes the protection against APP processing (Mehmedbasic, et al, 2015).

Two clones were isolated from each of these samples and tested via karyotyping to ensure that no chromosomal abnormalities arose during the reprogramming process (see below). Additional clones and a pool of clones were also cryopreserved for additional potential clones of each line for the future.

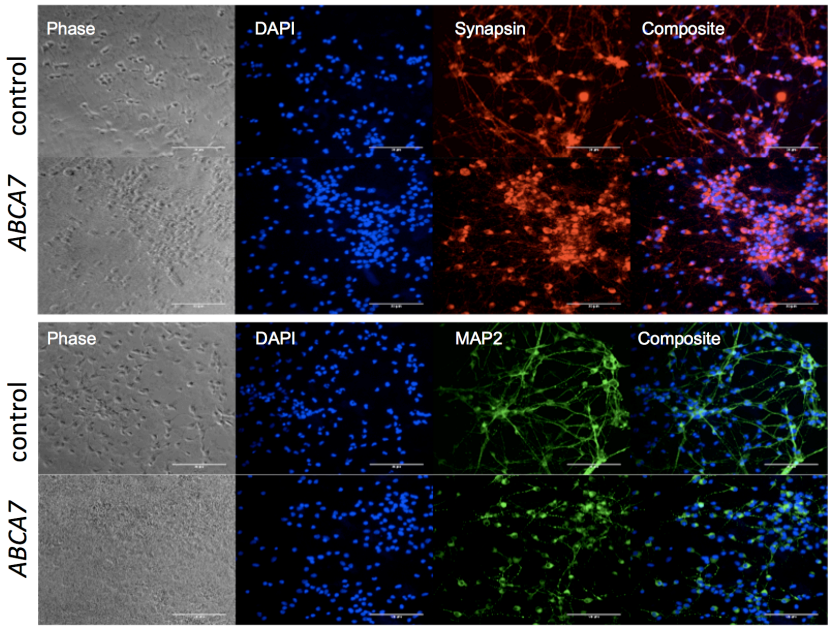


Karyotype analysis from iPSC clones of derived from a female with a deletion in the *ABCA7* (left), the individual with the variant in *SORL1* (middle), and an individual that carries an alteration in the *SEC16A* gene (right). All karyotypes are normal.



We were able to determine that the *ABCA7* and *SORL1* lines both expressed these genes during neuronal differentiation. We isolated RNA from these patient-derived lines and performed RT-PCR in order to generate cDNA. The *ABCA7* cDNA was generated from peripheral blood mononuclear cells (PBMCs), as well as day 23 neuronal precursor cells (NPCs). This cDNA was then amplified across the 44 base pair deletion, which presented with a visually detectable double band in the patient, which is a heterozygote expressing both a wild type and mutated form of the gene (A). For the *SORL1* lines, we were able to determine that the gene was being expressed, but the agarose gel did not permit resolution of the single base pair deletion (B). Finally, we were able to detect via immunocytochemistry (ICC) that day 35 neurons were expressing the ABCA7 protein (C), demonstrating that phenotypes detected at this relatively early time point could be related to disruption of this protein.

Neurons derived from the *ABCA7* lines were plated at day 35 for ICC and fixed at day 40. Both the control and AD case demonstrated that they were expressing neuronal markers including the broad neuronal marker synapsin as well as the more mature neuronal marker MAP2.

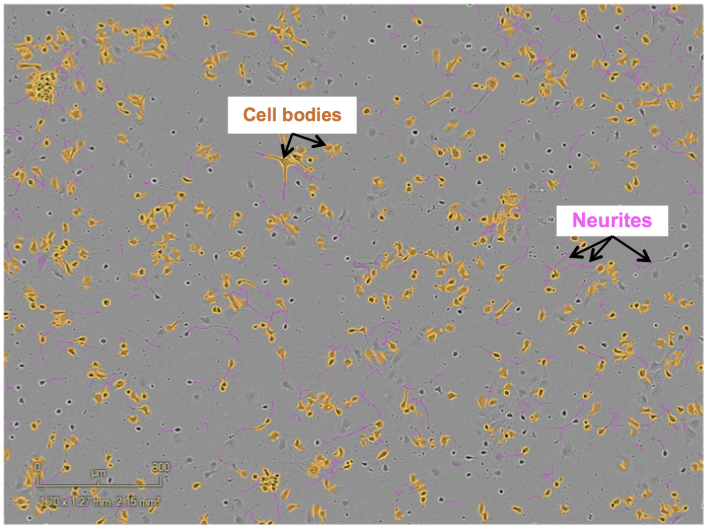


We have optimized cellular function assays that measure beta amyloid, tau, neurite growth, and rate of apoptosis. ELISA assays are used to analyze of the different amyloid beta species (Ab40 and Ab42) thought to be pathogenic in AD from the cell culture supernatant of the neurons. We have tested one of the *ABCA7* patient lines that carries the 44 base pair deletion in parallel with an ethnically matched (AA) control line from a neurologically normal individual at a relatively early neuronal stage (day 40-45) as well as an older culture from the same lines. At both time points, neurons

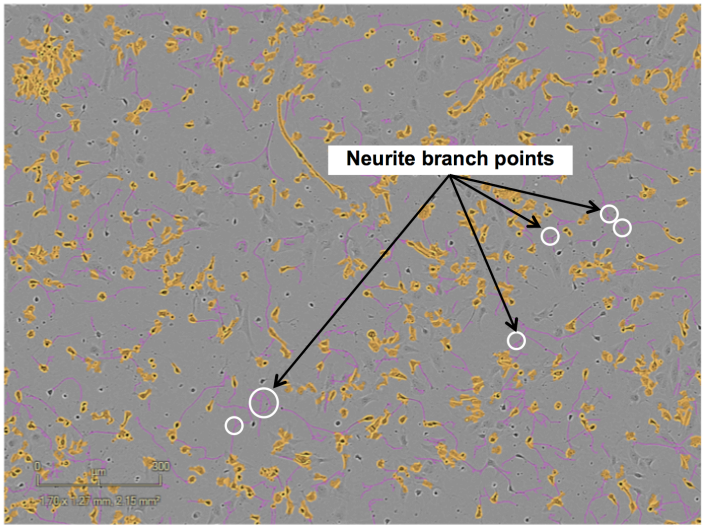
from the patient line had a higher level of amyloid beta 40 compared to neurons generated from the control individual. Furthermore, amyloid beta is being secreted at higher levels in both lines as the cells age.

In addition, morphological measures of axon and synapse formation were assessed in differentiating neuronal cultures using the IncuCyte ZOOM live cell imaging system (Essen BioScience). The IncuCyte ZOOM supports high content phase contrast, green fluorescence and red fluorescence imaging modes. The differentiating neurons were plated into triplicate wells of a 24-well plate coated with poly-D-lysine, laminin, and fibronectin. The IncuCyte ZOOM can capture images of growing cultures. The results of 4 fields of view/well from each well were analyzed to measure the cell body area (shown in yellow-orange below) as well as identify the neurites (purple) and the neurite branch points (encircled). The measurements for axon length/cell body area and branch points/cell body area were analyzed by the Neurotrack software.

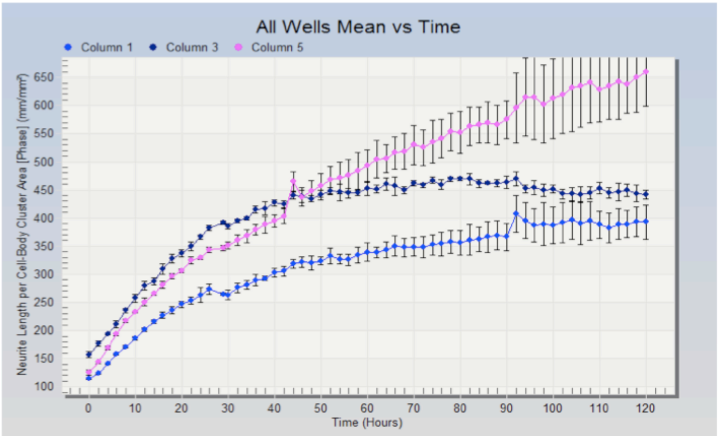
4 hours

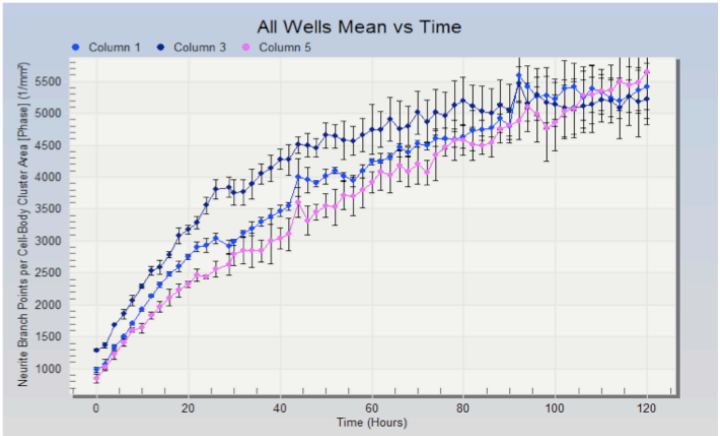


28 hours



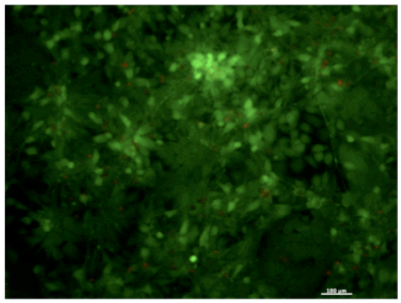
Preliminary results with one *ABCA7* line (shown in royal blue below) compared to two African American control lines lacking the 44 base pair deletion is shown below for days 35-40. The cells were placed into the IncuCyte ZOOM and analyzed for 5 days with imaging occurring every 4 hours. It appears that neurite length in the AD case, when normalized to the cell body area, may be reduced compared to controls (below, left). However, neurite branching does not appear to be different in the case compared to the control lines (below right).



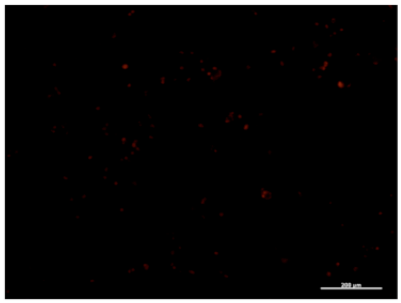


Furthermore, although the most consistent findings in AD-specific iPSC derived neurons has been increased levels of Ab42, Ab40, and phosphorylated tau, there have also been reports of neurons from AD patient-derived iPSC lines were more susceptible to cell death (Duan, et al, Mol. Neurodegener, 2014). Therefore, we sought to analyze the health and viability of the cultures throughout the differentiation process using the LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies). Love cells stain green, while dead cells are marked red. Initial experiments performed on day 35 cells under the normal growth conditions demonstrated that

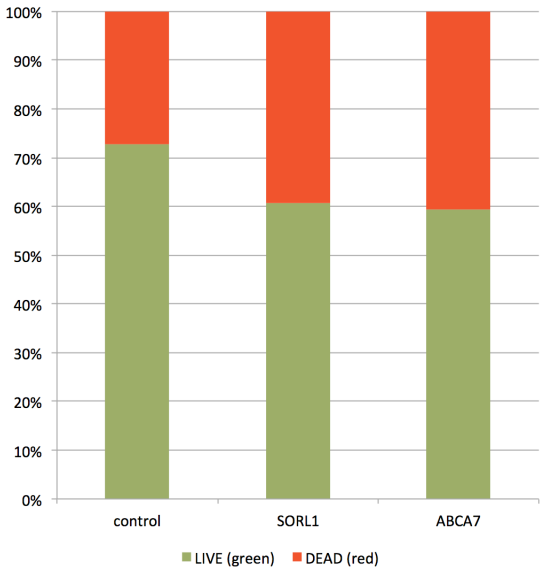
there may be an increase in cell death in neurons generated from *ABCA7* and *SORL1* AD patients. In the future, we may modify the assay to further treat the cells by adding deregulated calcium (treatment with ionomycin) or glutamate exposure (excitotoxicity) to the cultures.



LIVE = **green**
Calcein AM



DEAD = **red**
Ethidium homodimer-1



KEY RESEARCH ACCOMPLISHMENTS:

- Publication of the manuscript describing the *SORL1* alterations that we identified in EOAD and LOAD families, and how some of these individuals exhibit parkonsonian features (Cuccaro, et al, 2016).
- Manuscript describing the results from the whole exome sequencing is currently under revision following initial reviewer comments from *JAMA Neurology* (Kunkle, et al).
- iPSC lines have been generated from 2 AD individuals with the *SORL1* nonsense change, 2 AD individuals with the *ABCA7* 44 base pair deletion (rs142076058), and a single AD individual with the *SEC16A* alteration.
- Confirmed that *ABCA7* RNA and protein is being made in the iPSC and neuronal precursor cells
- Verified that *SORL1* RNA is being expressed in the iPSC and day 35 neurons
- Optimized the following functional assays:
    - Amyloid beta quantification of pathogenic species in the culture supernatant via ELISA
    - Live measuring of growing neurons and measuring neurons development through the neurite length and number of branch points (a sign of neuron maturity)
    - LIVE/DEAD apoptosis staining through immunocytochemistry
- Initial results indicate that the *ABCA7* deletion carrying patients may have decrease in neuronal maturity, as measured by shorter neurite length in day 35-40 neurons compared to controls.
- Preliminary data shows that both the ABCA7 and SORL1 alterations may make the cells more vulnerable to cell death, as shown through the LIVE/DEAD assay.
- A R01 grant entitled "*Genetic Epidemiology of Early-Onset Alzheimer's disease in Caribbean Hispanics and non-Hispanic Whites*" was awarded to Drs. Beecham (University of Miami) and Reitz (Columbia University). This grant will utilize whole genome sequencing of early onset AD families in a set of multiplex families using extreme phenotyping designs to increase statistical power by creating more homogeneous and genetically loaded populations, leading to the potential to reveal genetic risk factors and mechanisms difficult to identify in more heterogeneous datasets.

REPORTABLE OUTCOMES:

Manuscripts:

SORL1 mutations in early- and late-onset Alzheimer disease. Cuccaro, *et al*. Published in Neurol Genet. 2016 Oct 26;2(6):e116. (Appendix VII)

Whole-exome sequencing identifies novel candidate genes for early-onset Alzheimer disease. *Currently under revision for JAMA Neurology.*

Whole-exome sequencing of Hispanic families identifies novel candidate genes for early-onset Alzheimer disease. *In preparation.*

Presentations:

Alzheimer's Association International Conference (AAIC), Toronto, Canada, July 24-28, 2016:

Derek M. Dykxhoorn, Holly N. Cukier, Brian W. Kunkle, Badri N. Vardarajan, Sophie Rolati, Kara L. Hamilton-Nelson, Martin A. Kohli, Patrice L. Whitehead, Derek J. Van Booven, Rosalyn Lang, Lindsay A. Farrer, Michael L. Cuccaro, Jeffery M. Vance, John R. Gilbert, Gary W. Beecham, Eden R. Martin, Regina M. Carney, Richard P. Mayeux, Gerald D. Schellenberg, Goldie S. Byrd, Jonathan L. Haines, Margaret A. Pericak-Vance, and the Alzheimer Disease Genetics Consortium. ***ABCA7* Frameshift Deletion Associated with Alzheimer's Disease in African Americans.** Platform Presentation. (Appendix I)

Brian W. Kunkle, PhD, MPH[1], Badri N. Vardarajan, PhD[2], Adam C. Naj, PhD[3], Holly N. Cukier, PhD[1], Derek M Dykxhoorn, Ph.D.[1], Sophie Rolati, MS[1], Patrice L. Whitehead, BS[1], Regina M. Carney, MD[1], Michael L. Cuccaro, PhD[1], Jeffery M. Vance, MD, PhD[1], Alzheimer's Disease Genetics Consortium[4], Lindsay A. Farrer, PhD[5], Jonathan L. Haines, PhD[6], Gerard D Schellenberg, PhD[3], Eden R. Martin, PhD[1], Christiane Reitz, MD PhD[2], Gary W. Beecham, PhD[1], Richard Mayeux, MD, MSc[2]and Margaret A. Pericak-Vance, PhD[1] **Identification of Novel Candidate Genes for Early-Onset Alzheimer Disease through Integrated Whole-Exome Sequencing and Exome Chip Array Association Analysis.** Poster Presentation. (Appendix II)

Cuccaro ML, Carney RM, Kunkle BW, **Cukier HN**, Vardarajan BN, Whitehead PL, Mayeux R, St. George-Hyslop P, Pericak-Vance MA. **Pathogenic *SORL1* Mutations and Parkinsonian Features in Alzheimer's disease.** Poster Presentation. (Appendix III)

American Society for Human Genetics (ASHG), Vancouver, Canada, October 18-22, 2016:

Cukier HN, Gross SP, Kunkle BW, Rolati S, Hamilton-Nelson KL, Dombroski BA, Vardarajan BN, Whitehead PL, Lang R, Martin ER, Beecham GW, Farrer LA, Cuccaro ML, Vance JM, Mayeux RP, Gilbert JR, Carney RM, Byrd GS, Haines JL, Schellenberg GD, Pericak-Vance MA, Dykxhoorn DM. **Patient-derived iPSC model of an *ABCA7* deletion associated with Alzheimer disease.** Platform Presentation. (Appendix IV)

Cuccaro ML, Carney RM, Zhang Y, Bohm C, Kunkle BW, Vardarajan BN, Whitehead PL, Cukier HN, Mayeux R, St. George-Hyslop P, Pericak-Vance MA. ***Pathogenic SORL1* mutations in Alzheimer's Disease**. Poster Presentation. (Appendix V)

International Conference on Alzheimer's & Parkinson's Diseases (AD/PD), Vienna, Austria, Mar 29-Apr 2, 2017:

Cukier HN, Gross SP, Kunkle BW, Rolati S, Hamilton-Nelson KL, Whitehead PL, Martin ER, Beecham GW, Cuccaro ML, Vance JM, Mayeux RP, Gilbert JR, Byrd GS, Haines JL, Schellenberg GD, Pericak-Vance MA, Dykxhoorn DM. **Patient-derived iPSC model of an *ABCA7* deletion associated with Alzheimer disease in African Americans.** Poster Presentation. (Appendix VI)

CONCLUSION:

Mutations in *APP*, *PSEN1* and *PSEN2* lead to familial EOAD and accounting for 60-70% of familial EOAD and ~11% of EOAD overall, leaving the majority of genetic risk for this form of Alzheimer disease unexplained. We performed Whole-Exome Sequencing (WES) on 55 individuals in 19 Caribbean Hispanic EOAD families and 51 Non-Hispanic White EOAD cases previously screened negative for *APP*, *PSEN1* and *PSEN2* to search for rare variants contributing to risk for EOAD. Variants were filtered for segregating, conserved and functional rare variants (MAF<0.1%) assuming both autosomal and X-linked dominant models. We have identified and published or submitted manuscripts on variants identified in these analyses. We continue to identify candidate risk genes for EOAD, including an endocytic gene, *PSD2*, which we find to be significant in an analysis of EOAD exome chip association data from the ADGC. A comparison of these results to our EOAD WES sequencing identified two NHW cases with rare, damaging, missense variants in the *PSD2* gene. We also are following up our most promising results in iPSC analysis and have ascertained patient samples from the Hussman Institute Human Genomics (HIHG) cohort and the Columbia University cohort with genetic variants in SORL1, SEC16A, and ABCA7 for these analyses. PBMCs have been reprogrammed into iPSC from the patient samples and validated for pluripotency and karyotype. In addition, we have optimized how to measure the secreted amyloid beta 40 and 42 products, internal tau, measure apoptosis rate with the LIVE/DEAD assay and quantified how the young neurons grow and mature with the incucyte zoom machine. Initial results demonstrate that the ABCA7 lines may produce more toxic forms of amyloid beta and have a slower rate of neuronal maturity, as measured by shorter neurite length in day 35-40 neurons compared to controls. Furthermore, preliminary data shows that both the *ABCA7* and *SORL1* alterations may make the cells more vulnerable to cell death, as shown through the LIVE/DEAD assay.

APPENDICES:

Appendix I: AAIC 2016 Platform Presentation

***ABCA7* Frameshift Deletion Associated with Alzheimer's Disease in African Americans**

Derek M. Dykxhoorn, Holly N. Cukier, Brian W. Kunkle, Badri N. Vardarajan, Sophie Rolati, Kara L. Hamilton-Nelson, Martin A. Kohli, Patrice L. Whitehead, Derek J. Van Booven, Rosalyn Lang, Lindsay A. Farrer, Michael L. Cuccaro, Jeffery M. Vance, John R. Gilbert, Gary W. Beecham, Eden R. Martin, Regina M. Carney, Richard P. Mayeux, Gerald D. Schellenberg, Goldie S. Byrd, Jonathan L. Haines, Margaret A. Pericak-Vance, and the Alzheimer Disease Genetics Consortium.

Background: The *ATP-binding cassette, sub-family A (ABC1), member 7* (*ABCA7*) gene has been implicated as a risk factor in Alzheimer's disease (AD) in African American (AA), Asian, and non-Hispanic white (NHW) populations. However, the effect in African Americans is significantly stronger, comparable to that found in *APOE ϵ4*. While some rare loss-of-function variants in *ABCA7* were identified in NHW populations that may contribute to AD pathogenicity, potentially pathogenic variants have yet to be reported in AA populations.

Methods: We performed custom next generation sequencing across the *ABCA7* region on 40 AA individuals with AD and 37 control AA carrying the previously reported risk allele, rs115550680. Custom capture was performed across a 150 kb genomic area encompassing *ABCA7* and sequenced on the Illumina Hiseq. Data processing was performed with Casava, GATK, and BWA, and deletions were identified with Pindel. Our top variants were confirmed by Sanger sequencing and validated in two AA cohorts – HIHG (cases: 539, controls: 529) and ADGC (cases: 687, controls: 1,062). Whole exome sequencing was performed on 19 Caribbean Hispanic multiplex AD families (46 cases and 6 unaffected relatives). iPSC lines were developed from AA AD individuals bearing the *ABCA7* deletion which were differentiated into cortical neurons and functionally characterized.

Results: A 44 base pair deletion (rs142076058) was identified in all 77 risk genotype carriers. The deletion was significantly associated with disease (p=0.0002, OR=2.13 [95% CI:1.42-3.20]) in the HIHG cohort and replicated in the ADGC cohort ($p=1.414x10^{-5}$, OR=1.81 [95% CI:1.38-2.37]). The deletion is common in AA cases (15.2%) and AA controls (9.74%), but in only 0.12% of our NHW cohort. Whole exome sequencing of multiplex, Caribbean Hispanic families identified the deletion co-segregating with disease in a large sibship. The deleted allele produces a stable, detectable RNA strand and is predicted to result in a frameshift mutation (p.Arg578Alafs) that could interfere with protein function. IPSC-derived cortical neurons bearing the *ABCA7* deletion produced elevated levels of pathogenic β-amyloid production compared to controls.

Conclusions: The deletion in *ABCA7* could represent an ethnically-specific pathogenic alteration in Alzheimer's disease that results in impaired APP processing and increased toxic β-amyloid production.

Appendix II: AAIC 2016 Poster Presentation

Identification of Novel Candidate Genes for Early-Onset Alzheimer Disease through Integrated Whole-Exome Sequencing and Exome Chip Array Association Analysis

Brian W. Kunkle, PhD, MPH[1], Badri N. Vardarajan, PhD[2], Adam C. Naj, PhD[3], Holly N. Cukier, PhD[1], Derek M Dykxhoorn, Ph.D.[1], Sophie Rolati, MS[1], Patrice L. Whitehead, BS[1], Regina M. Carney, MD[1], Michael L. Cuccaro, PhD[1], Jeffery M. Vance, MD, PhD[1], Alzheimer's Disease Genetics Consortium[4], Lindsay A. Farrer, PhD[5], Jonathan L. Haines, PhD[6], Gerard D Schellenberg, PhD[3], Eden R. Martin, PhD[1], Christiane Reitz, MD PhD[2], Gary W. Beecham, PhD[1], Richard Mayeux, MD, MSc[2]and Margaret A. Pericak-Vance, PhD[1]

(1)University of Miami, Miami, FL, USA, (2)Columbia University, New York, NY, USA, (3)University of Pennsylvania, Philadelphia, PA, USA, (4)University of Pennsylvania School of Medicine, Philadelphia, PA, USA, (5)Boston University, Boston, MA, USA, (6)Case Western Reserve University, Cleveland, OH, USA

Abstract Text:
Background: Mutations in APP, PSEN1 and PSEN2lead to familial early-onset Alzheimer disease (EOAD). These mutations account for ~11% of EOAD overall, leaving the majority of genetic risk for the most severe form of AD unexplained. Methods: We performed whole-exome sequencing (WES) on 53 Non-Hispanic White EOAD cases screened negative for APP, PSEN1, and PSEN2 to search for rare EOAD risk variants. Variant filtering for missense and loss-of-function (LOF) rare variants (MAF<0.1%) was performed, and filtered variants present on the Illumina exome chip array were tested in a cohort of 1,292 EOAD cases (Age-at-onset<65) and 5,625 controls (Age≥65) from the Alzheimer's Disease Genetics Consortium (ADGC). As no LOF filtered variants were on the chip, we assessed these variants by using both the lowest variant P-value per gene and gene-based SKAT-O results. Rare LOF variants were prioritized for testing if they were in 2+ cases and in haploinsufficient genes (Haploinsufficient Score≥50). Results: 1,803 rare missense variants identified in the WES sample were available for testing in in the exome chip study. 70 of these variants were nominally associated with EOAD, with the strongest signals in the neuropeptide NPPB (OR=11.24, P=0.003), and NDST2 (OR=14.95, P=0.001), a processor of heparin sulfate, a molecule with potential importance in Aβ formation. Assessment of the 70 nominally significant genes containing these variants for consistent dysregulation (3+ expression studies) in AD using AlzBase prioritized 11 genes with strong potential for involvement in EOAD, including the APP interector ADRA1A (OR=3.60, P=0.032). One variant in the LOAD GWAS gene RIN3 (OR=6.94, P=0.009) also was of interest. Testing of 30 LOF genes revealed several associations that approached significance including a splicing variant in CACNA1G (OR=4.93, P=0.002), a gene associated with cognitive decline and age-related production of Aβ, a stopgain in CENPF (OR=11.46, P=0.004), a gene involved in endolysosomal transport and amyloid plaque generation, and a frameshift in the gene TDP2 (Gene P=0.006), for which homozygous LOF mutations cause neurodegeneration with epilepsy. Conclusions: Testing of candidate WES EOAD risk variants and genes in the ADGC EOAD exome chip study identified several genes with potential roles in AD pathogenesis.

Appendix III: AAIC 2016 Poster Presentation

Pathogenic *SORL1* Mutations and Parkinsonian Features in Alzheimer's disease

Cuccaro ML, Carney RM, Kunkle BW, Cukier HN, Vardarajan BN, Whitehead PL, Mayeux R, St. George-Hyslop P, Pericak-Vance MA.

Background
The *sortilin-related receptor LDLR class A repeats containing* (*SORL1*) gene has been implicated in both early and late onset Alzheimer's disease (AD). Located on chromosome 11q23.2-q24.2, *SORL1* plays a key role in the differential sorting of the amyloid precursor protein (APP) and regulation of the amyloid-β (Aβ) production. We describe the clinical and molecular findings among individuals with AD who present with *SORL1* mutations.

Methods
SORL1 changes were initially identified by whole exome sequencing in 50 early onset AD families with at least one case with onset <60 years of age. The clinical consequences associated with *SORL1* mutations were characterized based on extensive clinical reviews of medical records. Functional studies were completed to evaluate Aβ production and APP trafficking associated with *SORL1*mutations. Constructs were generated using human SORL1-MYC pcDNA3.1. Site directed mutagenesis was used to insert the T588I and T2134IM mutations verified by sequencing, and either the wild type or mutant constructs transfected into HEK293 cells expressing the Swedish APP mutant (APPsw). Cell culture and transfection followed previously described standard protocols. Aβ, Western blot, and co-immunoprecipitation assays were performed.

Results
A novel *SORL1* T588I change was identified four individuals with AD from one family; a second family was found to carry the T2134 alteration in three of four AD cases. Two cases with the *SORL1* T588I alteration also presented with Parkinsonian features; one case with the T2134M variant was identified postmortem to have Lewy bodies. Examination of unrelated patients with late onset AD (onset >65 years) identified four additional individuals with either *SORL1* A528T or T947M alterations who also presented with Parkinsonian features. Functional studies demonstrate that the variants weaken the interaction of *SORL1with* full-length APP, resulting in altered levels of Aβ and interfering with APP trafficking.

Conclusions
The findings from this study support an important role for *SORL1* mutations in AD pathogenesis by way of altering Aβ levels and interfering with APP trafficking. In addition. the results point to a potential association of *SORL1* in the manifestation of Parkinsonian features among individuals with AD, thus expanding the pheotypic spectrum of *SORL1* mutations.

Appendix IV: ASHG 2016 Platform Presentation

Patient-derived iPSC model of an *ABCA7* deletion associated with Alzheimer disease

H.N. Cukier[1,2], S.P. Gross[1], B.W. Kunkle[1], B.N. Vardarajan[3], S. Rolati[1], K.L. Hamilton-Nelson[1], P.L. Whitehead[1], B.A. Dombroski[4], R. Lang[5], L.A. Farrer[6], M.L. Cuccaro[1,7], J.M. Vance[1,2,7], J.R. Gilbert[1,7], G.W. Beecham[1,7], E.R. Martin[1,7], R.M. Carney[1,7], R. Mayeux[3], G.D. Schellenberg[4], G.S. Byrd[5], J.L. Haines[8], M.A. Pericak-Vance[1,2,7], D.M. Dykxhoorn[1,7]

1) John P. Hussman Institute for Human Genomics, University of Miami, Miller School of Medicine, Miami, FL.; 2) Department of Neurology, University of Miami, Miller School of Medicine, Miami, FL.; 3) The Taub Institute for Research on Alzheimer's Disease and the Aging Brain, Gertrude H. Sergievsky Center, Departments of Neurology, Psychiatry, and Epidemiology, College of Physicians and Surgeons, Columbia University, New York, NY.; 4) Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.; 5) Department of Biology, North Carolina A&T State University, Greensboro, NC.; 6) Departments of Medicine, Neurology, Ophthalmology, Genetics & Genomics, Epidemiology, and Biostatistics, Boston University, Boston, MA.; 7) Dr. John T. Macdonald Foundation Department of Human Genetics, University of Miami, Miller School of Medicine, Miami, FL.; 8) Department of Epidemiology and Biostatistics, Institute for Computational Biology, Case Western Reserve University School of Medicine, Cleveland, OH.

The *ATP-binding cassette, sub-family A (ABC1), member 7* (*ABCA7*) gene has been implicated as a risk factor in Alzheimer disease (AD) across populations including African American (AA), Asian, and non-Hispanic white (NHW). However, the effect is significantly stronger in AA than in the Asian and NHW populations. While some rare loss-of-function *ABCA7* variants have been identified in NHWs, we recently identified a relatively common 44 base pair deletion (rs142076058) in African Americans significantly associated with disease ($p=1.414x10^{-5}$, OR=1.81 [95% CI:1.38-2.37], Cukier, et al, 2016). The deleted allele is predicted to produce a frameshift mutation (p.Arg578Alafs) resulting in a truncated protein. To further understand the mechanism by which the *ABCA7* deletion may be acting, iPSC lines were developed from two unrelated AA AD individuals bearing the deletion, as well as two gender, aged, and ethnical matched control individuals. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and reprogrammed by transducing with Sendai virus vectors expressing the Yamanaka factors. Two clones from each individual were generated, validated for pluripotency, and karyotyped to ensure that no gross chromosomal abnormalities were present. The iPSC lines were cultured first as embroid bodies, then neural rosettes, and finally as a monolayer in media supplemented with small molecules to promote cortical neuronal differentiation. RNA purified from both PBMCs and neuronal cells from AD individuals produce a stable RNA transcript from the *ABCA7* allele with the deletion. iPSC-derived cortical neurons from cases and controls were then examined by an enzyme-linked immunosorbent assay (ELISA) to measure levels of pathogenic β40-amyloid at day 45 and 95 of differentiation. While higher levels of β40-amyloid were found in the older neurons (day 95) compared to younger neurons (day 45), the AD cases had higher levels of β40-amyloid compared to controls. In conclusion, this deletion in *ABCA7* may represent an ethnic-specific pathogenic alteration in AD resulting in impaired APP processing and increased production of toxic β-amyloid production.

Appendix V: ASHG 2016 Poster Presentation

Pathogenic *SORL1* mutations in Alzheimer's Disease

Cuccaro ML, Carney RM, Zhang Y, Bohm C, Kunkle BW, Vardarajan BN, Whitehead PL, Cukier HN, Mayeux R, St. George-Hyslop P, Pericak-Vance MA.

The *sortilin-related receptor LDLR class A repeats containing* (*SORL1*) gene has been implicated in both early and late onset Alzheimer's disease (AD). Located on chromosome 11q23.2-q24.2, *SORL1* plays a key role in differential sorting of the amyloid precursor protein (APP) and regulation of amyloid-β (Aβ) production. We describe the clinical and molecular findings among individuals with AD and *SORL1* mutations.

SORL1 changes were identified by whole exome sequencing in 50 early onset AD families which contained at least one case with AD onset less than 60 years of age. The clinical consequences associated with SORL1 mutations were characterized based on clinical reviews of medical records. Functional studies were completed to evaluate (Aβ) production and APP trafficking associated with SORL1 mutations. Constructs were generated using human SORL1-MYC pcDNA3.1. Site directed mutagenesis was used to insert the T588I and T2134IM mutations verified by sequencing, and either the wild type or mutant constructs transfected into HEK293 cells expressing the Swedish APP mutant (APPsw). Cell culture and transfection followed previously described standard protocols. Aβ, Western blot, and co-immunoprecipitation assays were performed.

A novel *SORL1* T588I change was identified in four individuals with AD from one EOAD family, two of whom also presented with Parkinsonian features. A second EOAD family was found to carry the T2134 alteration in three of four AD cases; one case with the T2134M variant was identified postmortem to have Lewy bodies. Two individuals, one with AD and one with MCI, were found to carry a p.Cys1431fs mutation in *SORL1*. Functional studies demonstrate that the variants weaken the interaction of *SORL1* with full-length APP, resulting in altered levels of Aβ and interfering with APP trafficking. Finally, a review of unrelated cases from a previously published study of *SORL1* mutations in late onset AD (Vardarajan 2015) identified four individuals with either *SORL1* A528T or T947M alterations who also presented with Parkinsonian features.

The findings from this study support an important role for *SORL1* mutations in AD pathogenesis by way of altering Aβ levels and interfering with APP trafficking. In addition, the results point to a potential association of *SORL1* in the manifestation of Parkinsonian features among individuals with AD, expanding the phenotypic spectrum of *SORL1* mutations.

Appendix VI: AD/PD 2017 Poster Presentation

Patient-derived iPSC model of an *ABCA7* deletion associated with Alzheimer disease in African Americans

Cukier HN, Gross SP, Kunkle BW, Rolati S, Hamilton-Nelson KL, Whitehead PL, Martin ER, Beecham GW, Cuccaro ML, Vance JM, Mayeux RP, Gilbert JR, Byrd GS, Haines JL, Schellenberg GD, Pericak-Vance MA, Dykxhoorn DM.

Objective: The *ATP-binding cassette, sub-family A (ABC1), member 7* (*ABCA7*) gene has been implicated as a risk factor in Alzheimer's disease (AD) in African American (AA) and non-Hispanic white (NHW) populations. However, the effect is significantly stronger in African Americans than in the Asian and NHW populations. We recently reported a relatively common 44 base pair deletion in AA significantly associated with disease ($p=1.414x10^{-5}$, Cukier, et al, 2016). The deleted allele is predicted to produce a frameshift mutation (p.Arg578Alafs) resulting in a truncated protein.

Methods: To further understand the mechanism by which the *ABCA7* deletion may be acting, induced pluripotent stem cells (iPSC) lines were developed from the blood of two unrelated AA AD individuals bearing the deletion, as well as two aged and ethnically matched neurologically normal control individuals.

Results: Each iPSC line generated was validated for pluripotency and karyotyped. The iPSC lines were cultured as embroid bodies, then neural rosettes, and finally as a monolayer to promote cortical neuronal differentiation. RNA from the AD individuals have demonstrated that a stable RNA transcript is being produced from the *ABCA7* deletion allele. iPSC-derived cortical neurons were then examines by ELISA to measure levels of pathogenic β40-amyloid at day 45 and 95 of differentiation. While higher levels of β40-amyloid were found in the older neurons compared to younger neurons, the AD cases had higher levels of β40-amyloidcompared to controls.

Conclusions: This deletion in ABCA7 may represent an ethnically-specific pathogenic alteration in AD resulting in impaired APP processing and increase production of toxic β-amyloid production.

SORL1 mutations in early- and late-onset Alzheimer disease

OPEN

Michael L. Cuccaro, PhD*
Regina M. Carney, MD*
Yalun Zhang, PhD
Christopher Bohm, PhD
Brian W. Kunkle, PhD
Badri N. Vardarajan, PhD
Patrice L. Whitehead, BS
Holly N. Cukier, PhD
Richard Mayeux, MD, MSc
Peter St. George-Hyslop, MD
Margaret A. Pericak-Vance, PhD

Correspondence to Dr. Pericak-Vance: mpericak@med.miami.edu

ABSTRACT

Objective: To characterize the clinical and molecular effect of mutations in the sortilin-related receptor (*SORL1*) gene.

Methods: We performed whole-exome sequencing in early-onset Alzheimer disease (EOAD) and late-onset Alzheimer disease (LOAD) families followed by functional studies of select variants. The phenotypic consequences associated with *SORL1* mutations were characterized based on clinical reviews of medical records. Functional studies were completed to evaluate β-amyloid (Aβ) production and amyloid precursor protein (APP) trafficking associated with *SORL1* mutations.

Results: *SORL1* alterations were present in 2 EOAD families. In one, a SORL1 T588I change was identified in 4 individuals with AD, 2 of whom had parkinsonian features. In the second, an SORL1 T2134 alteration was found in 3 of 4 AD cases, one of whom had postmortem Lewy bodies. Among LOAD cases, 4 individuals with either SORL1 A528T or T947M alterations had parkinsonian features. Functionally, the variants weaken the interaction of the SORL1 protein with full-length APP, altering levels of Aβ and interfering with APP trafficking.

Conclusions: The findings from this study support an important role for *SORL1* mutations in AD pathogenesis by way of altering Aβ levels and interfering with APP trafficking. In addition, the presence of parkinsonian features among select individuals with AD and *SORL1* mutations merits further investigation. ***Neurol Genet* 2016;2:e116; doi: 10.1212/NXG.0000000000000116**

GLOSSARY

AAO = age at onset; **Aβ** = β-amyloid; **AD** = Alzheimer disease; **APP** = amyloid precursor protein; **APPsβ** = APP soluble β-secretase; **APPsw** = Swedish APP mutant; **EOAD** = early-onset Alzheimer disease; **ER** = endoplasmic reticulum; **FL-APP** = full-length APP; **HIHG** = John P. Hussman Institute for Human Genomics; **LOAD** = late-onset Alzheimer disease; **PD** = Parkinson disease; ***SORL1*** = sortilin-related receptor; **WES** = whole-exome sequencing.

Alzheimer disease (AD) is the leading cause of dementia in the elderly.[1] Multiple genes have been implicated in risk for both late-onset Alzheimer disease (LOAD; onset >65 years of age) and early-onset Alzheimer disease (EOAD; onset <65 years of age)[2] including the sortilin-related receptor (*SORL1*) gene. Located on chromosome 11q23.2-q24.2, *SORL1* influences the differential sorting of the amyloid precursor protein (APP) and regulation of β-amyloid (Aβ) production, making it biologically plausible for AD risk.[3–9]

Compelling evidence for the involvement of *SORL1* in AD comes from a large meta-analysis of >30,000 individuals, which confirmed that variants in *SORL1* are associated with AD risk.[10]

Supplemental data at Neurology.org/ng

*These authors contributed equally to this work.

From the John P. Hussman Institute for Human Genomics (M.L.C., R.M.C., B.W.K., P.L.W., H.N.C., M.A.P.-V.), University of Miami Miller School of Medicine, FL; Mental Health & Behavioral Science Service (R.M.C.), Bruce W. Carter VA Medical Center, Miami, FL; The Taub Institute for Research on Alzheimer's Disease and the Aging Brain (B.N.V., R.M.), Gertrude H. Sergievsky Center, Departments of Neurology, Psychiatry, and Epidemiology, College of Physicians and Surgeons, Columbia University, New York, NY; Tanz Centre for Research in Neurodegenerative Diseases and Department of Medicine (Y.Z., C.B., P.S.G.-H.), University of Toronto, Ontario, Canada; Cambridge Institute for Medical Research (P.S.G.-H.), Department of Clinical Neurosciences, University of Cambridge, United Kingdom; and Dr. John T. Macdonald Foundation Department of Human Genetics (M.L.C., M.A.P.-V.), Miller School of Medicine, University of Miami, FL.

Funding information and disclosures are provided at the end of the article. Go to Neurology.org/ng for full disclosure forms. The Article Processing Charge was paid by the authors.

Furthermore, whole-exome sequencing (WES) has identified potentially damaging *SORL1* mutations in patients with both EOAD and LOAD.[11,12] Of note, a WES study of a large EOAD cohort found a greater frequency of predicted damaging missense *SORL1* variants in cases vs controls, with this effect enriched among cases with a positive family history.[13] Clearly, rare coding variants in *SORL1* are tied to risk for EOAD and LOAD. Finally, while *SORL1* mutations have been reported in multiple patients with AD, there has been little investigation of clinical phenotypes beyond dementia and age at onset (AAO) among these individuals.

For this study, we examined well-characterized EOAD families using WES to discover AD risk genes. Our efforts focused on clinical characterization of individuals with *SORL1* alterations and investigation of the functional effect of the identified *SORL1* alterations in a series of gene overexpression experiments.

METHODS

Standard protocol approvals, registrations, and patient consents. All participants ascertained for this study gave written informed consent prior to their inclusion. If an individual was not competent to give consent, the immediate next of kin or a legal representative provided written consent on their behalf. All participants were ascertained using a protocol that was approved by the appropriate Institutional Review Board. Oversight of this study falls under the University of Miami Institutional Review Board #20070307.

Sources of participants. EOAD families were ascertained as part of a larger study on AD genetics whose participants were enrolled under protocols previously described.[14,15] Individuals were ascertained for this study after they provided informed consent at the John P. Hussman Institute for Human Genomics (HIHG) at the University of Miami Miller School of Medicine (Miami, FL). The majority of these families were self-reported non-Hispanic whites (N = 47); the remaining families were self-reported African Americans (N = 3). Clinical data from cognitively impaired individuals, including any that changed affection status, were evaluated by the HIHG AD clinical staff which includes a psychiatrist, neurologist, and neuropsychologist. Familial EOAD cases were defined as AAO <65 years of age. As reported in previous studies, AAO was defined as the age at which an individual or family historian reported onset of significant cognitive problems that interfered with normal activity, or the AAO of problems as documented in the medical record.[15] All affected individuals met the internationally recognized standard NINCDS-ADRDA criteria.[16,17] The cognitive status of participants was measured using either the Mini-Mental State Examination[18] or the Modified Mini-Mental State.[19]

Patients with LOAD (N = 151) were part of a study investigating coding mutations in *SORL1* in AD.[11] These participants were drawn from a larger study of AD genetics restricted to Caribbean Hispanics. All affected individuals were of Caribbean Hispanic ancestry. All participants were assessed using standard clinical examinations and cognitive testing as described elsewhere.[20] For this study, we reviewed the clinical records of participants who had *SORL1* mutations to assess for possible features of Parkinson disease (PD) or more broadly, parkinsonism.

WES and variant calling. All samples were prepared using DNA extracted from the blood. Genomic DNA was then sheared and processed using the SureSelect Human All Exon 50 Mb v4 capture kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol at the HIHG Center for Genome Technology. After capture, the DNA was tested for uniform enrichment of targets via quantitative PCR. Sequencing was then performed on the Illumina HiSeq2000 at 2× 150 bp paired-end cycles at 40–50× on target depth. Exomes were sequenced to sufficient depth to achieve a minimum threshold of 80% of coding sequence covered with at least 15 reads, based on UCSC hg19 "known gene" transcripts. The mean depth of coverage across *SORL1* was 68.

Sequencing data from the Illumina HiSeq2000 were processed using an established semiautomated pipeline. Initial image files were processed using the Firecrest module (Illumina, San Diego, CA) to determine cluster intensities and noise. After initial quality control, BWA-ELAND and CASAVA v1.9 were used for realignment to the human genome version hg19. Results from BWA and CASAVA are then fed into additional software packages (CLC Genomics Workbench and GenomeStudio) for secondary analysis, visualization of the called variants, and browsing of consensus reads.[21,22] Genotype calling was performed with GATK Unified Genotyper. Variants were then normalized using BCFTools.[23] Single nucleotide polymorphisms with read depth <6, variant quality score log odds ratio <0, and Phred-scaled likelihood score <100 were removed from further analysis. Variants were filtered to identify alterations that were likely to be damaging (missense, splicing, stop-gain, stop-loss, and insertion/deletions) in Gencode v19, NCBI RefSeq, or Ensembl gene annotations.[24,25] Variants were screened to determine whether they occurred in a known or suspected EOAD gene (*APP*, *GRN*, *MAPT*, *PSEN1*, *PSEN2*, *SORL1*, and *TREM2*). Minor allele frequencies were obtained from the Exome Aggregation Consortium.[26]

Cloning of *SORL1* variants. Site-directed mutagenesis was used to generate the SORL1 T588I and SORL1 T2134IM mutation constructs using human SORL1-MYC pcDNA3.1 as a backbone according to the manufacturer's instructions as previously published.[3,11,27–30] Sequencing was used to verify mutant constructs. Cell culture and transfection followed previously described standard protocols.[3,11,27–30]

Aβ, Western blot, and co-immunoprecipitation assays. Aβ assays were measured by sandwich ELISA assay in culture medium from stably transfected HEK293 cells expressing the Swedish APP mutant (APPsw) and either wild-type SORL1 or mutant SORL1 as previously described.[3,11,27–30] Cell surface biotinylation was performed using 1 mg/mL Sulfo-NHS-LC-Biotin (Sigma-Aldrich, St. Louis, MO) for 20 minutes at 4°C to prevent internalization. Cells were then washed and lysed, and biotinylated proteins were precipitated with NeutrAvidin beads (Thermo Fisher Scientific, Waltham, MA). Western blot band intensities were measured with ImageJ software and samples normalized to the wild-type control. Co-immunoprecipitation was performed after cell lysis in 1% CHAPSO buffer,[3] using G Plus beads with 2 μg mouse monoclonal anti-c-MYC antibody for the immunoprecipitation of SORL1-myc, immunoblotted with anti-C-terminal APP antibody (Ab365), and anti-C-terminal SORL1 (S9200). Western blot band intensities were

measured with ImageJ software. Full-length (FL) APP coprecipitated with c-MYC antibody was quantified and normalized to the amount of immunoprecipated SORL1 as previously described.[3,11,27–30]

Statistical analyses. Statistical analyses were performed using Graphpad statistical software (graphpad.com/guides/prism/5/user-guide/prism5help.html?using_tour_overview.htm; GraphPad Prism 5). Analysis of variance and *t* tests were used to analyze statistical difference, followed by Bonferroni correction (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

RESULTS *SORL1* variants in EOAD families. WES identified 10 individuals with *SORL1* mutations in 2 unrelated EOAD families (table 1, figure 1). Neuropathology results were available for 1 affected individual. The first family, number 191, has 6 individuals with the predicted damaging SORL1 T588I mutation (rs752726649; C>T); all 4 affected individuals for whom DNA was available were found to carry this variant. These 4 affected individuals had AAOs that ranged from 59 to 82 years. While the progressive cognitive decline of each individual was consistent with dementia, individuals 104 and 111 had also parkinsonian features. Individual 104 began to show cognitive impairment at age 82. On examination, he demonstrated tremor at rest, hypophonia, micrographia, masked facial expression, smaller steps on gait, and overall bradykinesia. Chart review indicated that these symptoms were levodopa/carbidopa responsive. Imaging revealed white matter changes and moderate cerebral atrophy, and EEG was remarkable for a loss of alpha waves. Individual 111 had the earliest AAO in the family 191 at age 59, with diminished memory function in all domains, clinically judged to most likely represent EOAD. When seen by research staff at age 70, the individual was noted to exhibit parkinsonian features. This presentation was confounded by several years of treatment with haloperidol, a typical antipsychotic agent that can cause parkinsonian side effects. Two unaffected individuals in family 191 also carried the SORL1 T588I mutation. These individuals were last examined at ages 81 and 84 years, respectively. Individuals 116 and 9004 demonstrated a normal cognitive and physical examination.

The second family, number 1240 (table 1 and figure 1), contains 3 affected individuals with the SORL1 T2134M mutation (rs142884576; C>T). These 3 affected individuals had AAOs that ranged from 55 to 84 years. While the clinical examinations revealed no motor abnormalities, there was autopsy evidence for Lewy bodies in individual 1, with the earliest AAO in the family at 55 years. Neuropathologic diagnosis of individual 1 was indicative of Braak & Braak stage IV tangles and limbic Lewy bodies. In addition, 1 individual (119) demonstrated progressive cognitive decline consistent with AD without the T2134M SORL1 mutation. This individual had an AAO of 76 years. Finally, there was 1 unaffected individual (113) with this T2134M SORL1 mutation who was last examined at 79 years of age.

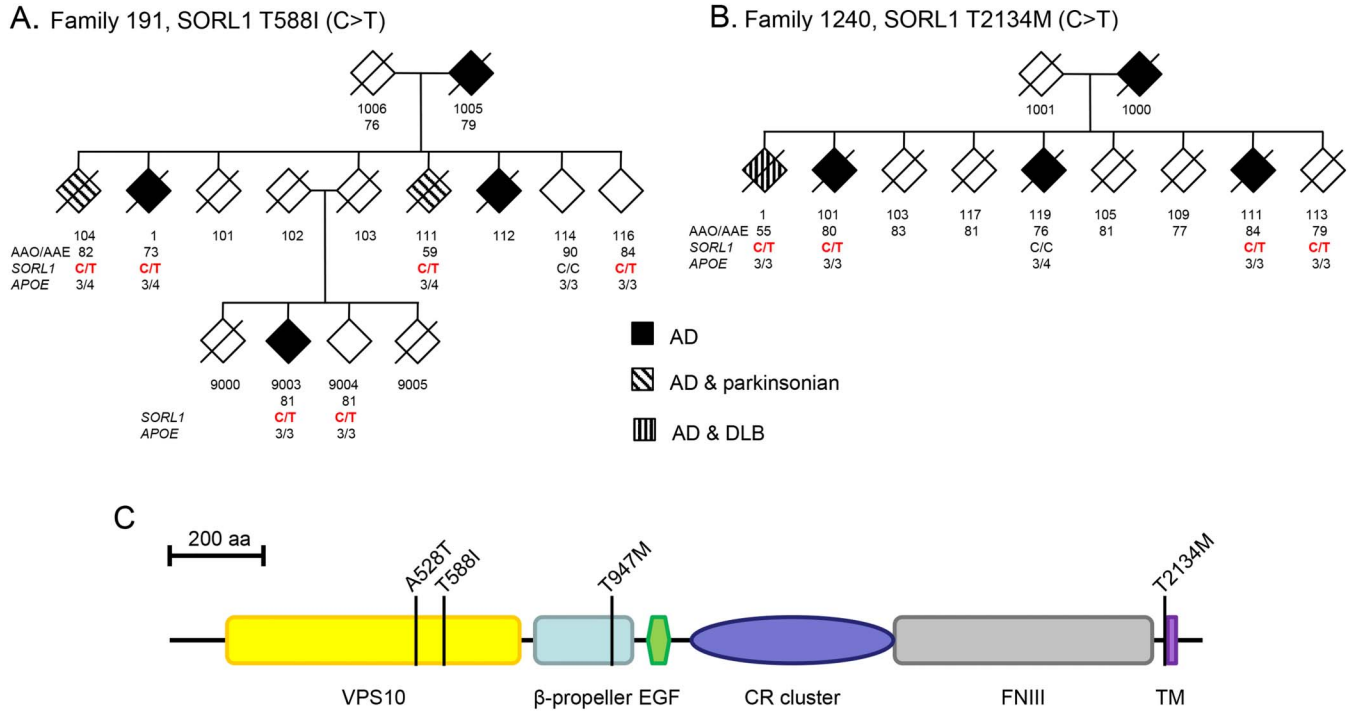
Parkinsonian features in patients with LOAD with *SORL1* variants. Given the clinical results from these 2 EOAD families, we examined in greater depth the clinical status of previously reported patients with *SORL1* changes.[11] Review of clinical history and physical examination data identified 4 additional AD individuals, all with LOAD (no neuropathology results were available), and who had evidence of

Table 1 Clinical features in early-onset AD families with *SORL1* variants

Family-individual	Sex	Affection status	Additional features	AAO/AAE	Base pair position (hg38 assembly)	Nucleotide	Amino acid	dbSNP number[a]	*APOE* status
191-1	M	Dementia AD	—	73	121543625	C>T	T588I	rs752726649	3/4
191-104	F	Dementia AD	Parkinsonian	82	121543625	C>T	T588I	rs752726649	3/4
191-111	M	Dementia AD	Parkinsonian	59	121543625	C>T	T588I	rs752726649	3/4
191-114	M	—	—	90	—	—	—	—	3/3
191-116	M	—	—	84	121543625	C>T	T588I	rs752726649	3/3
191-9003	M	Dementia AD	—	81	121543625	C>T	T588I	rs752726649	3/3
191-9004	M	—	—	81	121543625	C>T	T588I	rs752726649	3/3
1240-1	M	Dementia AD	DLB, no PD	55	121627591	C>T	T2134M	rs142884576	3/3
1240-101	F	Dementia AD	—	80	121627591	C>T	T2134M	rs142884576	3/3
1240-111	F	Dementia AD	—	84	121627591	C>T	T2134M	rs142884576	3/3
1240-113	M	—	—	79	121627591	C>T	T2134M	rs142884576	3/3
1240-119	F	Dementia AD	—	76	—	—	—	—	3/4

Abbreviations: AAE = age at examination; AAO = age at onset; AD = Alzheimer disease; DLB = dementia with Lewy bodies; PD = Parkinson disease; SNP = single nucleotide polymorphism.
[a] Minor allele frequencies (MAF): rs752726649 global MAF = 8.2×10^{-6}; rs142884576 global MAF = 2.2×10^{-4}.

Figure 1 **Pedigrees of the early-onset Alzheimer disease families and SORL1 protein diagram**



(A and B) Pedigrees of the early-onset Alzheimer disease (EOAD) families and SORL1 protein diagram. Affected individuals are solid black while those presenting with parkinsonian features are patterned. Below each individual number is either the age at onset (AAO, for affected individuals) or the age at last examination (AAE, for unaffected individuals). For family 191, the *SORL1* variant is present in all affected individuals examined. In family 1240, the variant occurs in 3 of 4 cases evaluated. (C) Diagram of SORL1 protein (2214 total amino acids) indicating the location of principal domains and the variants identified in the EOAD families (T588I and T2134M) and the late-onset Alzheimer disease individuals (A528T and T947M). AD = Alzheimer disease; VPS10 = vesicular protein sorting 10 domain; CR = complement type repeat domains; EGF = epidermal growth factor; FNIII = fibronectin type III repeats; TM = transmembrane region; DLB = dementia with Lewy bodies; *SORL1* = sortilin-related receptor.

parkinsonian features (table 2). The *SORL1* mutations in these 4 individuals were distinct from those identified in the first 2 families. Specifically, 3 individuals which we previously reported carry a common variant at A528T (rs2298813A>G). Clinically, these individuals were diagnosed with both AD and PD and had ages of AD onset ranging from 78 to 84 years. The fourth individual had a different previously reported missense T947M variant (rs143571823, C>T). This individual had a clinical diagnosis of AD and parkinsonism with an age of AD onset at 90 years.

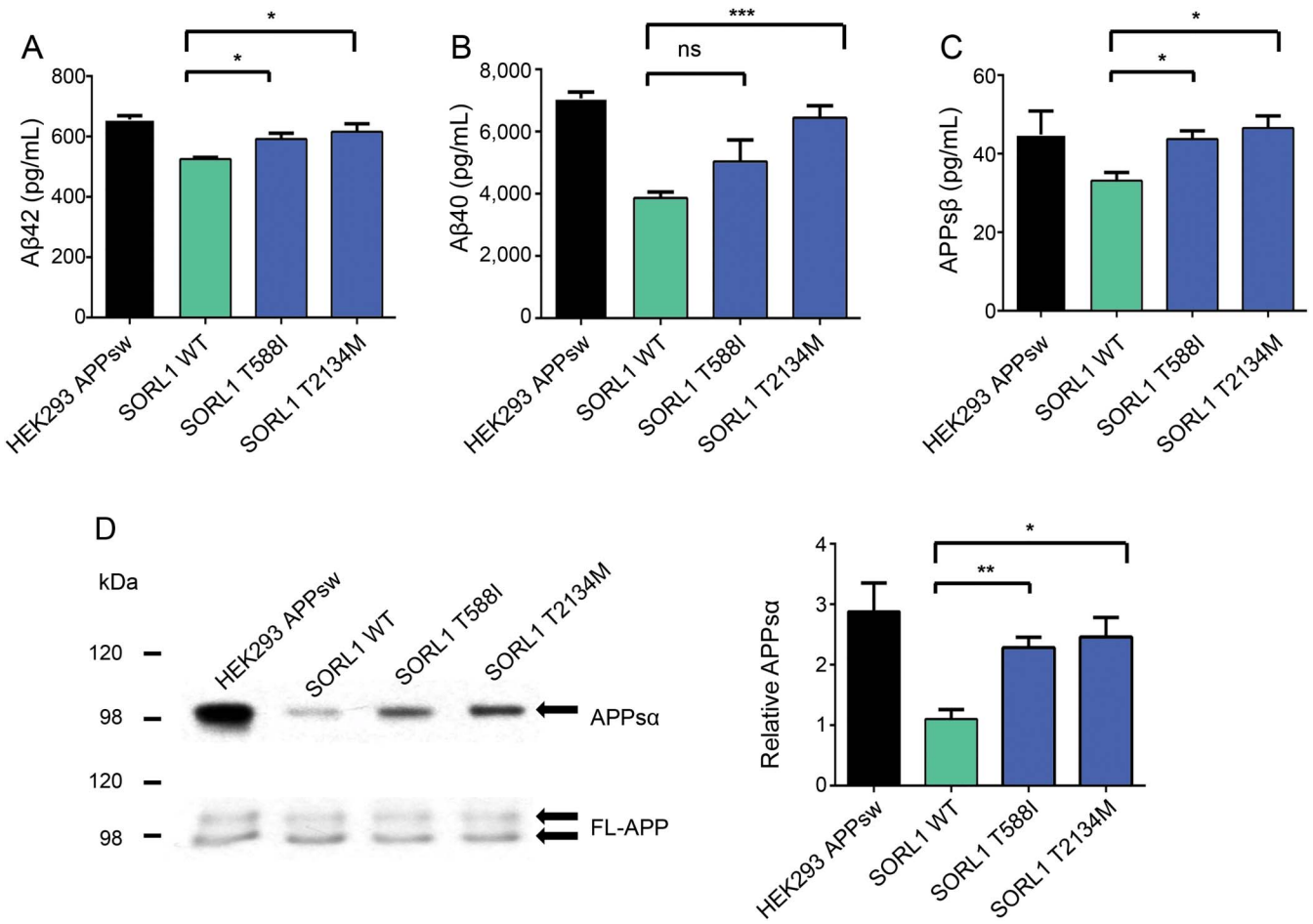
***SORL1* variants alter Aβ levels and APP trafficking.** Next, we examined the functional consequences of the SORL1 T588I and T2134M alterations identified in the EOAD families; the variants identified in the LOAD individuals (A528T and T947M) were previously assessed and reported.[11] To determine the effects on Aβ production by these *SORL1* variants, Aβ42 and Aβ40 levels were measured in conditioned media collected from cultured HEK293 cells expressing equivalent levels of wild-type SORL1 protein, SORL1 T588I, or SORL1 T2134M. Both mutants increased Aβ42 secretion compared with the control (T588I: 113% ± 1.6% and T2134M: 117% ± 5.1%, $p < 0.05$, figure 2A). Overexpression of SORL1 T2134M also increased Aβ40 secretion (167% ± 9.9%, $p < 0.001$, figure 2B). While the

Table 2 **Clinical features in late-onset AD individuals with *SORL1* variants**

Family-individual	Sex	Affection status	Additional features	AAO	Base pair position (hg38 assembly)	Nucleotide	Amino acid	dbSNP number[a]	*APOE* status
216-9	M	Dementia AD	Parkinsonian	78	121522975	A>G	A528T	rs2298813	2/4
920-10	F	Dementia AD	Parkinsonian	83	121522975	A>G	A528T	rs2298813	3/4
1280-18	F	Dementia AD	Parkinsonian	90	121558767	C>T	T947M	rs143571823	3/4
1731-1	M	Dementia AD	Parkinsonian	84	121522975	A>G	A528T	rs2298813	3/3

Abbreviations: AAO = age at onset; AD = Alzheimer disease; SNP = single nucleotide polymorphism.
[a] Minor allele frequencies (MAF): rs2298813 MAF = 0.072; rs143571823 MAF = 0.0013.

Figure 2 ***SORL1* mutants' overexpression increases β-amyloid secretion**



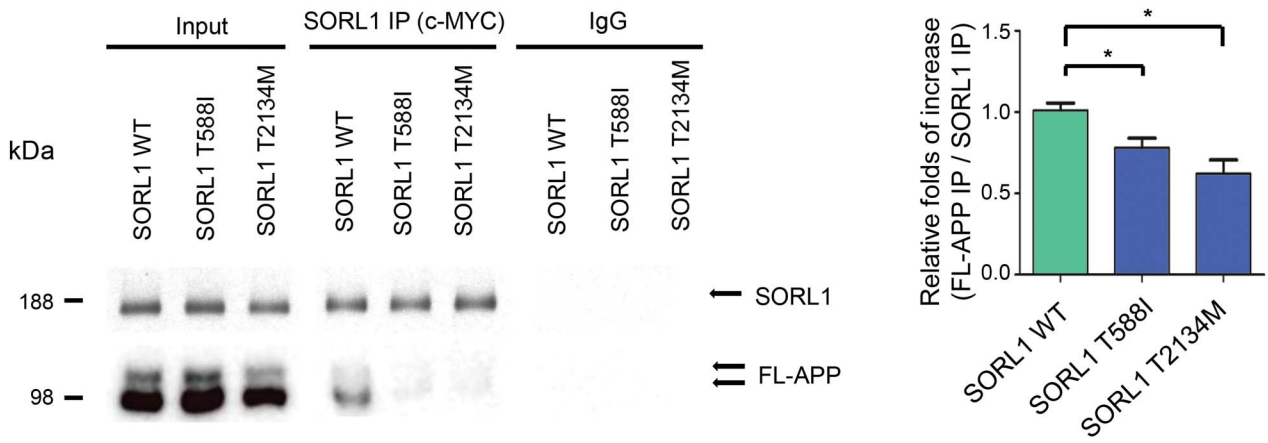
(A–C) Secreted β-amyloid 40 (Aβ40), Aβ42, and amyloid precursor protein soluble β-secretase (APPsβ) were measured from culture medium in stable HEK293 cells expressing the APP Swedish mutant (HEKsw) together with either wild-type *SORL1* or mutant *SORL1*. Error bars represent standard error of the mean (SEM). ***$p < 0.001$, *$p < 0.05$, ns, not significant, n = 3 independent replications. (D) Western blot was performed to detect APP soluble α-secretase (APPsα) from cultured media. Bar graphs were normalized to control. **$p < 0.01$, n = 3 independent replications, and error bars represent the SEM. Aβ = β-amyloid; FL-APP = full-length APP; *SORL1* = sortilin-related receptor.

SORL1 T588I alteration trended toward an increase of Aβ40 secretion in cells, it did not reach statistical significance (131% ± 17.6%, figure 2B).

SORL1 has been proposed to modulate the post-translational biology of APP at several intracellular sites including during transport out of the Golgi and during re-entry and recycling from the cell surface. To examine further the effect of these *SORL1* mutants on APP trafficking, we measured APP soluble β-secretase (APPsβ) secretion in a conditioned medium.[31] Both mutations caused an increase in APPsβ secretion compared with the wild-type *SORL1* (T588I: 132% ± 6.3%, $p < 0.05$; T2134M: 140% ± 9.4%, $p < 0.05$, figure 2C). Both mutations also increased production of the soluble α-secretase cleavage product compared with control cells (T588I: 207% ± 15.8%, $p < 0.01$; T2134M: 223% ± 29.6%, $p < 0.05$, figure 2D). These observations suggest that in the presence of these *SORL1* mutants, APP is neither retained efficiently in the Golgi nor effectively retrieved from the cell surface into recycling pathways. This could result in additional APP lingering at the cell surface. This hypothesis was supported by surface biotinylation experiments which revealed that both *SORL1* mutants increased the amount of surface APP compared with the control (T588I: 143% ± 13.1%, $p < 0.05$; T2134M: 138% ± 7.5%, $p < 0.05$, figure e-1 at Neurology.org/ng).

***SORL1* variants decrease APP binding.** To understand the mechanism by which these *SORL1* mutants might alter APP trafficking at the cell surface, we next measured levels of SORL1 protein at the cell surface. The T588I variant showed essentially normal levels of SORL1 both at the cell surface and in total cell lysates (~87% ± 13.1% of control value, figure e-1). However, while the T2134M mutant showed normal levels of total cellular SORL, there were decreased amounts of surface SORL1 (~25%, $p < 0.05$, figure e-1B).

Figure 3 **Both *SORL1* mutants have a reduced binding affinity to APP**



SORL1 was immunoprecipitated from cell lysates with a c-MYC antibody, and the amount of coprecipitated full-length amyloid precursor protein (FL-APP) was measured by densitometry of the anti-APP immunoreactive bands on the Western blot of the SORL1 immunoprecipitation products. *$p < 0.05$, **$p < 0.01$, n = 3 replications, and error bars represent the SEM. IgG = immunoglobulin G; IP = immunoprecipitated; *SORL1* = sortilin-related receptor.

Previous work by us and others have demonstrated that SORL1 directly binds APP and regulates its sorting into secretory, endocytic, or recycling pathways.[3,4,11,27,32–38] To assess whether the SORL1 T588I and T2134M mutations might alter the binding affinity of SORL1 to APP, we immunoprecipitated SORL1 from whole cell lysates using an anti-myc antibody directed to the myc epitope on the exogenous SORL1 protein. This strategy circumvents possible risk that the *SORL1* mutants might alter binding affinity of anti-SORL1 antibodies, or that endogenous SORL1 might be pulled down in addition, to overexpressed SORL1 in the mutant APPsw cell lines. We then measured the amount of FL-APP that co-immunoprecipitated with the myc-tagged SORL1 proteins and expressed the binding as a normalized ratio of the abundance of coprecipitated FL-APP relative to the abundance of immunoprecipitated SORL1. Both mutations caused reductions in APP binding (T588I: ~77.1% ± 5.8%, $p < 0.05$; T2134M: ~61.5% ± 8.3%, $p < 0.05$, figure 3).

DISCUSSION In this study, we identified *SORL1* alterations in EOAD families thus confirming previously reported studies showing a role for *SORL1* in risk for EOAD. Furthermore, we presented functional evidence that these *SORL1* alterations are pathogenic.

Evidence for functional consequences of *SORL1* mutations is scant. However, the evidence shown here suggests that the variants identified in the EOAD families, SORL1 T588I and T2134, weaken the interaction of SORL1 with FL-APP. This can culminate in excessive APP accumulating at the cell surface either due to failure of the mutant SORL1 to slow trafficking of APP to the cell surface[39] or failure of mutant SORL1 to retrieve FL-APP into the retromer-recycling endosome pathway.[3,4,11,27,32–38] Our result agrees with prior work which suggests that some *SORL1* mutants cause reduced trafficking of the mutant SORL1 protein from the endoplasmic reticulum (ER)/Golgi network to the cell surface.[11] The resulting misdirection of more APP into the late endosome pathway exposes the APP to β-secretase and γ-secretase cleavage, with the consequent increase in Aβ production, especially Aβ42. Intriguingly, but consistent with prior work, our data suggest that the molecular mechanisms underlying this common overall effect differ between the 2 variants. Thus, the T2134M mutant, which is located close to the transmembrane domain (figure 1), appears to disrupt trafficking of SORL1 to the cell surface, presumably due to its removal from the ER-Golgi secretory pathway by the ER quality control systems which remove misfolded proteins. In contrast, the T588I mutant survives the ER quality control mechanisms, but appears to be less efficient than wild-type SORL1 in binding to APP. The molecular mechanism for the reduced binding of T588I is unclear, but may relate to subtle changes in the fold of the extracellular domain of SORL1 such that putative APP-binding sites in VPS10 and/or in complement type repeat domains.[39,40] Crucially, while they may have different underlying molecular mechanisms, the net effect of both mutations is the same.

A secondary finding in our study was the observation of additional clinical features beyond AD among select individuals with *SORL1* alterations. These clinical findings, based on extensive clinical reviews, included clinical Parkinson-related features and neuropathology-proven Lewy bodies without clinical parkinsonism. While these findings point to

a potential association between *SORL1* alterations and a broader spectrum of neurodegenerative disorders, it is important to note that these clinical features were not present in all individuals with *SORL1* alterations and may simply represent features of coincidental sporadic PD.

The results from this study demonstrate that select *SORL1* variants present in EOAD and LOAD alter Aβ levels and interfere with APP trafficking. In addition, we observed parkinsonian features among some EOAD/LOAD individuals with *SORL1* alterations. These clinical findings should be viewed cautiously but suggest the need for exploration of the additional phenotype consequences of *SORL1* alterations beyond dementia.

AUTHOR CONTRIBUTIONS

M.L.C., R.M.C., B.W.K., and M.A.P.-V. conceived and designed the experiments. M.L.C., R.M.C., R.M., and M.A.P.-V. acquired and assessed participants. P.L.W. and H.N.C. performed custom capture sequencing and exome sequencing. B.W.K., H.N.C., and M.A.P.-V. analyzed the sequencing data. B.W.K. and M.A.P.-V. performed the statistical analysis. Y.Z., C.B., and P.S.G.-H. cloned the *SORL1* variants, performed all assays, and analyzed all resulting data. M.L.C. and R.M.C. drafted the manuscript. M.L.C., R.M.C., Y.Z., C.B., B.W.K., H.N.C., P.S.G.-H., and M.A.P.-V. edited the manuscript. The authors jointly discussed the experimental results over the course of the study. All authors read and approved the final manuscript.

ACKNOWLEDGMENT

The authors are grateful to the families and staff who participated in this study. In addition, they acknowledge Larry Deon Adams for his assistance in data management and Anthony Griswold for his help in bioinformatics.

STUDY FUNDING

This research was supported by grants from the National Institutes of Health (R01 AG027944 and R01 AG028786 to M.A.P.-V.; R01 AG019085 to J.L.H.; P20 MD000546 and R01 AG28786-01A1 to G.S.B.; U01-AG032984 and RC2-AG036528 to G.D.S., U24 AG026395, U24 AG026390, R01AG037212, R37 AG015473, and R01AG015473 to R.M.; R01 AG009029 to L.A.F.; U01-AG016976 to W.A.K.; U24-AG021886 to T.M.F.; R01AG009956 and RC2 AG036650 to K.H.; UO1 AG06781 and UO1 HG004610 to E.L.; 5R01AG20688 to M.D.F.; P50 AG005133 and AG030653 to I.K.; R01 AG1101, R01 AG030146, and RC2 AG036650 to D.A.E.; P30AG10161, R01AG15819, R01AG30146, R01AG17917, and R01AG15819 to D.A.B.; R01AG028786 to J.J.M.; R01AG22018 and P30AG10161 to L.L.B.; P50AG16574, R01 032990, and KL2 RR024151 to N.E.T. and N.R.G.R.; AG005138 to J.D.B.; P50 AG05681, P01 AG03991, and P01 AG026276 to A.M.G.; U24-AG041689 to L.S.W.; and U19 AG047133 and UF1AG047133); the Department of Defense (W81XWH-12-1-0013 to M.A.P.-V.); a joint grant from the Alzheimer's Association (SG-14-312644) and the Fidelity Biosciences Research Initiative to M.A.P.-V.; and the BrightFocus Foundation (A2011048 to M.A.P.-V.).

DISCLOSURE

Dr. Cuccaro has served on the editorial board of *Child Psychiatry & Human Development*. Dr. Carney, Dr. Zhang, Dr. Bohm, and Dr. Kunkle report no disclosures. Dr. Vardarajan has served on the scientific advisory board of, been a consultant of, and received research support from the Immuneering Corporation. Ms. Whitehead reports no disclosures. Dr. Cukier has served on the scientific advisory board of the General Direction for Scientific Research and Health Innovation (Italian Ministry of Health); and has received research support from NARSAD, the Hussman Foundation, and the Alzheimer's Association. Dr. Mayeux has received research support from the following grants: RO1 AG037212 Epidemiology of Biomarkers of Risk and Progression in Late onset Alzheimer's Disease; and RF1 AG015473 Genetic Studies of Alzheimer's Disease in Caribbean Hispanics. Dr. St. George-Hyslop has served on the scientific advisory board of the University of Lille (basic AD program) and has received research support from the Canadian Institutes of Health, the Medical Research Council, and Wellcome Trust. Dr. Pericak-Vance reports no disclosures. Go to Neurology.org/ng for full disclosures.

Received June 13, 2016. Accepted in final form September 23, 2016.

REFERENCES

1. Karch CM, Goate AM. Alzheimer's disease risk genes and mechanisms of disease pathogenesis. Biol Psychiatry 2015;77:43–51.
2. Alzheimer's Association. 2015 Alzheimer's disease facts and figures. Alzheimers Dement 2015;11:332–384.
3. Rogaeva E, Meng Y, Lee JH, et al. The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease. Nat Genet 2007;39:168–177.
4. Offe K, Dodson SE, Shoemaker JT, et al. The lipoprotein receptor LR11 regulates amyloid beta production and amyloid precursor protein traffic in endosomal compartments. J Neurosci 2006;26:1596–1603.
5. Lee JH, Barral S, Reitz C. The neuronal sortilin-related receptor gene SORL1 and late-onset Alzheimer's disease. Curr Neurol Neurosci Rep 2008;8:384–391.
6. Meng Y, Lee JH, Cheng R, St George-Hyslop P, Mayeux R, Farrer LA. Association between SORL1 and Alzheimer's disease in a genome-wide study. Neuroreport 2007;18:1761–1764.
7. Kolsch H, Jessen F, Wiltfang J, et al. Association of SORL1 gene variants with Alzheimer's disease. Brain Res 2009;1264:1–6.
8. Lee JH, Cheng R, Honig LS, Vonsattel JP, Clark L, Mayeux R. Association between genetic variants in SORL1 and autopsy-confirmed Alzheimer disease. Neurology 2008;70:887–889.
9. Yin RH, Yu JT, Tan L. The role of SORL1 in Alzheimer's disease. Mol Neurobiol 2015;51:909–918.
10. Reitz C, Cheng R, Rogaeva E, et al. Meta-analysis of the association between variants in SORL1 and Alzheimer disease. Arch Neurol 2011;68:99–106.
11. Vardarajan BN, Zhang Y, Lee JH, et al. Coding mutations in SORL1 and Alzheimer disease. Ann Neurol 2015;77:215–227.
12. Pottier C, Hannequin D, Coutant S, et al. High frequency of potentially pathogenic SORL1 mutations in autosomal dominant early-onset Alzheimer disease. Mol Psychiatry 2012;17:875–879.
13. Nicolas G, Charbonnier C, Wallon D, et al. SORL1 rare variants: a major risk factor for familial early-onset Alzheimer's disease. Mol Psychiatry 2016;21:831–836.
14. Naj AC, Jun G, Beecham GW, et al. Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. Nat Genet 2011;43:436–441.
15. Carney RM, Slifer MA, Lin PI, et al. Longitudinal follow-up of late-onset Alzheimer disease families. Am J Med Genet B Neuropsychiatr Genet 2008;147B:571–578.
16. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. Neurology 1984;34:939–944.

17. McKhann GM, Knopman DS, Chertkow H, et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association Workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 2011;7:263–269.
18. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state." A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 1975;12:189–198.
19. Teng EL, Chui HC. The modified mini-mental state (3MS) examination. J Clin Psychiatry 1987;48:314–318.
20. Vardarajan BN, Faber KM, Bird TD, et al. Age-specific incidence rates for dementia and Alzheimer disease in NIA-LOAD/NCRAD and EFIGA families: National Institute on Aging Genetics Initiative for Late-Onset Alzheimer Disease/National Cell Repository for Alzheimer Disease (NIA-LOAD/NCRAD) and Estudio Familiar de Influencia Genetica en Alzheimer (EFIGA). JAMA Neurol 2014;71:315–323.
21. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009; 25:1754–1760.
22. McKenna A, Hanna M, Banks E, et al. The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20: 1297–1303.
23. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 2011;27:2987–2993.
24. Raney BJ, Dreszer TR, Barber GP, et al. Track data hubs enable visualization of user-defined genome-wide annotations on the UCSC genome browser. Bioinformatics 2014; 30:1003–1005.
25. Kent WJ, Sugnet CW, Furey TS, et al. The human genome browser at UCSC. Genome Res 2002;12:996–1006.
26. Exome Aggregation Consortium. Analysis of protein-coding genetic variation in 60,706 humans. Nature 2016;536:285–291.
27. Reitz C, Tokuhiro S, Clark LN, et al. SORCS1 alters amyloid precursor protein processing and variants may increase Alzheimer's disease risk. Ann Neurol 2011;69: 47–64.
28. Bohm C, Seibel NM, Henkel B, Steiner H, Haass C, Hampe W. SorLA signaling by regulated intramembrane proteolysis. J Biol Chem 2006;281:14547–14553.
29. Hasegawa H, Sanjo N, Chen F, et al. Both the sequence and length of the C terminus of PEN-2 are critical for intermolecular interactions and function of presenilin complexes. J Biol Chem 2004;279:46455–46463.
30. Yu G, Nishimura M, Arawaka S, et al. Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. Nature 2000;407:48–54.
31. Vassar R, Bennett BD, Babu-Khan S, et al. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 1999; 286:735–741.
32. Jacobsen L, Madsen P, Jacobsen C, Nielsen MS, Gliemann J, Petersen CM. Activation and functional characterization of the mosaic receptor SorLA/LR11. J Biol Chem 2001;276: 22788–22796.
33. Andersen OM, Reiche J, Schmidt V, et al. Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein. Proc Natl Acad Sci USA 2005;102:13461–13466.
34. Dodson SE, Andersen OM, Karmali V, et al. Loss of LR11/SORLA enhances early pathology in a mouse model of amyloidosis: evidence for a proximal role in Alzheimer's disease. J Neurosci 2008;28:12877–12886.
35. Hermey G. Targeting amyloid precursor protein. Ann Neurol 2011;69:8–10.
36. Fjorback AW, Seaman M, Gustafsen C, et al. Retromer binds the FANSHY sorting motif in SorLA to regulate amyloid precursor protein sorting and processing. J Neurosci 2012;32:1467–1480.
37. Lane RF, Steele JW, Cai D, Ehrlich ME, Attie AD, Gandy S. Protein sorting motifs in the cytoplasmic tail of SorCS1 control generation of Alzheimer's amyloid-beta peptide. J Neurosci 2013;33:7099–7107.
38. Schmidt V, Sporbert A, Rohe M, et al. SorLA/LR11 regulates processing of amyloid precursor protein via interaction with adaptors GGA and PACS-1. J Biol Chem 2007; 282:32956–32964.
39. Mehmedbasic A, Christensen SK, Nilsson J, et al. SorLA complement-type repeat domains protect the amyloid precursor protein against processing. J Biol Chem 2015;290: 3359–3376.
40. Gustafsen C, Glerup S, Pallesen LT, et al. Sortilin and SorLA display distinct roles in processing and trafficking of amyloid precursor protein. J Neurosci 2013;33:64–71.

1. Budget Status

Provide financial information requested below. Add additional row for subaward greater than one and include the name of the organization receiving the subaward.

	This Quarter	**Cumulative**
Personnel	$27,855.50	$157,207.92
Fringe Benefits	$7,276.54	$56,666.71
Travel		$12,130.16
Equipment		
Supplies	$9,946.86	$63,244.37
Other	$26,323.47	$166,613.76
Subaward - Organization		$393,354.91
Subtotal	$64,125.83	$849,217.83
Indirect Costs	$33,986.67	$275,050.82
Fees		
TOTAL	$98,112.50	$1,124,268.65