UNIVERSITY OF CALIFORNIA SAN DIEGO

Cholesterol Regulates APP Processing in iPSC-Derived Human Neurons

A thesis submitted in partial fulfillment of the requirement
for the degree of Master of Science

in

Biology

by

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2019

The Thesis of Louie Wang as it is listed on UC San Diego Academic Records is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

2019

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
APP FL	Amyloid Precursor Protein Full Length
Aβ	Amyloid-beta
BACE 1	Beata(β)-secretase
CTF	Amyloid Precursor Protein C-Terminal Fragment
dNPCs	Differentiated Neural Progenitor Cells
hiPSCs	Human Induced Pluripotent Stem Cells
NPCs	Neural Progenitor Cells
NFTs	Neurofibrillary Tangles
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RP-dNPCs	Replated Differentiated Neural Progenitor Cells
sAPP	Soluble Amyloid Precursor Protein Fragment
WT	Wild Type

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ACKNOWLEDGEMENTS

First and foremost, I want to thank Dr. Lawrence Goldstein for accepting me into his lab in my junior undergraduate year and giving me a fantastic learning opportunity that improved my intellectual ability to practice science. I have learned so much about Alzheimer's disease, about doing tissue cultures and experimental procedures, about critically challenge data and results, and about research in general. I would also like to thank Dr. Brenda Bloodgood and Dr. James Cooke for taking the time to serve on my thesis committee.

Next, I want to thank Vanessa Langness, my research mentor for the past three years, for her patient training and continued support. Her mentorship was more than I ever expected and have taught me valuable lessons that are important for the rest of my career, and life.

I want to thank Dr. Angels Almenar-Queralt, Dr. Utpal Das, and Dr. Chao-Shun Yang, for helping me analyze my western blot results. I also want to thank Dr. Kenneth W. Henry for his help in my ongoing fight against mold issues in my incubator. Finally, I would like to all the members of the Goldstein lab, past and present, for their energy, positivity, and sometimes needed cakes and snacks.

ABSTRACT OF THE THESIS

Cholesterol regulates APP processing in iPSC-Derived Human Neurons

by

Louie Wang

Master of Science in Biology

University of California San Diego, 2019

Professor Lawrence Goldstein, Chair
Professor Brenda Bloodgood, Co-Chair

Alzheimer's Disease (AD) is a chronic neurodegenerative disease that is characterized by the formation of Amyloid-beta(Aβ) plaques in the brain. My project focuses on Amyloid Precursor Protein (APP) processing which generates Aβ. APP has a cholesterol-binding domain (Beel et al., 2010). We used CRISPR-CAS9 to induce mutations that abolish APP- cholesterol

binding in stem cells which were then differentiated to neurons. In the mutants that lack cholesterol binding, we observed decreased Aβ 42, increased β-CTF, and no change in APP full-length levels, when compared to wild type (WT) at baseline. In wild-type neurons treated with atorvastatin, we saw decreased Aβ 42, sAPPβ, and β-secretase, increased APP full length, and no change in β-CTF, Presenilin 1, and Presenilin 2 levels. Similar changes were observed in atorvastatin-treated mutants, however, the decrease in Aβ 42 and the increase in APP full length was blunted compared to atorvastatin treated wild-types. Our finding indicates that cholesterol regulates APP processing through mechanisms that are more complex than previously hypothesized. This observation can provide de novo insights to the relation of cholesterol and APP processing, with relevance to AD pathogenesis.

INTRODUCTION

Alzheimer's Disease

Alzheimer's Disease (AD) is a chronic neurodegenerative disease that usually begins with short term memory loss. AD slowly progresses to dementia and ultimately leads to death (Sarkar et al., 2019). AD is commonly characterized into two categories: sporadic AD (SAD), characterized by a late onset, and familial AD (FAD), characterized by an early onset in middle age. Mutations in one of three genes: amyloid precursor protein (APP), Presenilin 1(PSEN 1), and Presenilin 2(PSEN 2) cause FAD. FAD is inherited in a Mendelian fashion, with little influence from the environment. Most AD cases are sporadic and can be attributed to a variety of genetic and environmental risk factors (Barber. 2012). While many genetic risk factors have been associated with SAD, the apolipoprotein E (APOE) e4 allele is the major genetic risk factor. (Dorszewska et al., 2016). Although genetics plays a large role in the development of SAD, non-genetic factors are also crucial. Some of these factors like hypertension, diabetes, and obesity may have genetic components that have complex and unknown interaction mechanisms with other genes and environmental factors (Stozicka et al., 2007). The same review proposed that neurodegenerative diseases are not the result of single factor, but involve many genetic, epigenetic, and environmental events.

Alois Alzheimer first described the clinical symptoms and neuropathology of AD found in his patient Auguste Deter in 1906 (Foley, 2010). AD is characterized by the formation of Amyloid-beta (Aβ) plaques and neurofibrillary tangles containing hyperphosphorylated tau protein in the brain (O'Nuallain et al., 2010).

The first characteristic hallmark of AD is the amyloid plaque, composed of Aβ fragments which are hydrophobic peptides with varying lengths of amino acid residues (Rajnish et al, 2016). A study has shown that Aβ fragments that are 40 or 42 amino acids long have a strong

tendency to aggregate and form the clinically characteristic Aβ plaques (Esbjörner et al., 2014). This finding was one piece of data that formed and supported the amyloid cascade hypothesis of AD, which states that Aβ plaque toxicity is the cause of AD (Nalivaeva et al., 2019). The 42 amino acid fragment (Aβ42) was found to accumulate the most in Aβ plaques. (Suzuki et al., 1994). Aβ is derived from APP, a larger transmembrane protein. APP's physiological role remains unknown. Full Length APP (APP FL) is expressed in all cell types and can be cleaved in two pathways. In the non-amyloidogenic pathway, α-secretase cleaves APP to generate the soluble APP alpha fragment (sAPPα) and the α-C-terminal fragment (αCTF) which is further cleaved in its transmembrane region by γ-secretase, yielding the peptide p3. Aβ is not produced in this pathway. In the amyloidogenic pathway, APP is first cleaved by β-secretase to generate the soluble APP beta fragment (sAPPβ) and the β-C-terminal fragment (βCTF) which is further cleaved by γ-secretase and ultimately yields the Aβ peptides 40 and 42, which are thought to be neurotoxic and potentially one of the causes of AD (Kuperstein et al., 2010). At present, more than 160 mutations in three genes (APP, PSEN 1, PSEN 2) have been shown to cause FAD (Barber, 2012). Approximately 25 coding mutations on the APP gene cause AD (St George-Hyslop, 2000). Pathogenic APP mutations can either increase Aβ accumulation, increase the ratio of Aβ42/Aβ40 formation, or increase the propensity of Aβ to oligomerize into toxic species (Bertram et al., 2010; Bertram and Tanzi, 2012). One recent study has found that Aβ plaques are associated with tau pathogenesis, which is also a major focus for AD studies (He et al., 2017).

Neurofibrillary tangle formation, the second hallmark of AD, involves the microtubule-associated protein (MAP). Axonal microtubule-associated protein tau is a major type of MAP and is known to be involved in stabilizing and mediating axonal transport (Kevenaar and Hoogenraad, 2015). In AD, microtubule dissociated tau proteins aggregate into neurofibrillary

tangles (NFTs) and lead to cytoskeletal transportation disruptions and synaptic dysfunction (Zempel and Mandelkow., 2014). Tau has been found to be hyperphosphorylated in NFTs, and researchers have shown that hyperphosphorylation of tau (pTau) leads to increased aggregation (Bamburg et al., 2016).

Role of Cholesterol in Alzheimer's Disease

Cholesterol is a major component of the cell membrane and is responsible for cell structural support and modulating cell fluidity. Cholesterol can be synthesized by the liver and is consumed through the diet through foods containing animal and plant fats. Cholesterol is carried by different types of lipoproteins in the human bloodstream: chylomicrons, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). One of the early studies of how cholesterol is related to AD showed that rabbits fed a diet high in cholesterol later displayed a higher level of intraneuronal Aβ in the hippocampus compared with control rabbits (Sparks et al., 1994). Another study found that a diet high in cholesterol increased the brain deposition of Aβ in APP-transgenic mice (Refolo et al., 2000). These findings suggest that cholesterol demonstrates an influence on the production of Aβ. Cross-sectional and case-control studies examining the association between cholesterol levels and AD have found divergent results. One study reported that patients with AD had higher levels of total cholesterol compared to controls and other dementia patients (Lesser et al., 2001), while other studies did not find an association (Erkinjuntti et al., 1988, Bonarek et al., 2000). On the other hand, more studies reported lower levels of total cholesterol in patients with AD compared to non-demented controls (Kuusisto et al., 1997; Czyzewski et al., 2001). These mixed findings may reflect that the association of cholesterol with AD or dementia may vary

depending on when cholesterol is measured in the life-span and/or relative to the course of the disease.

Cholesterol in the brain is mainly synthesized in situ and independent from dietary sources (Jurevics and Morell, 1995). This finding reflects the constant need of cholesterol for myelin sheath and neuronal membrane, which would be difficult to keep at homeostasis if brain cholesterol can be easily exchanged with periphery cholesterol (Bjorkhem and Meaney, 2004). APOE is found on the surface of lipoproteins and its known functions include transporting lipids between cells (Koren, 1982) and coordinating cholesterol in the maintenance of myelin and neuronal membranes (Boyles et al., 1989). The APOE gene exists in three isoforms: e2, e3, and e4 (Strittmatter and Roses, 1995). Studies have shown that the inheritance of the APOE e4 allele is associated with increased brain cholesterol levels and an increased risk of developing AD compared to individuals with only the APOE e2 or APOE e3 alleles (Sing and Davignon, 1985, Hofman et al., 1997, Notkola et al., 1998). Although the mechanism of how APOE e4 increases the risk of AD is not fully understood, it was found that APOE e4 contributes to AD risk by decreasing the clearance of Aβ from the brain (Castellano et al., 2011). In the same study, it was also found that APOE e2 is associated with increased clearance of Aβ, in contrast to APOE e4. This suggests that APOE may play a role in the relationship between cholesterol and Aβ in the brain. Lowering brain cholesterol could potentially decrease Aβ levels.

In another study, cultured neurons were treated with statins which resulted in a reduction of total Aβ formation (Simons et al., 1998). Statins inhibit HMG (hydroxyl-3-methyl-glutaryl)-CoA reductase (HMG-CoA), the rate-limiting enzyme involved in cholesterol synthesis (Lennernas and Fager, 1997). One study demonstrated increased production of amyloid plaques in transgenic AD mice fed with a high cholesterol diet. The researchers found that statin

treatment (atorvastatin) reduced this production by approximately 50% (Refolo et al., 2000). With promising results found in vivo and in vitro, many epidemiology studies were conducted to test the use of statins to treat AD. Studies have found that human across all age groups tolerate statins well (4S(Scandinavian Simvastatin Survival Study)-Study-Group, 1994; Santanello et al., 1997; Feldman et al, 2010; Sano et al., 2011), and the main adverse effect, myopathy, occurs in only 0.1% of all cases and resolves with the termination of treatment (Maron et al., 2000). Using hospital records to compare the prevalence of AD between patients prescribed statins and other drugs, an epidemiology study reported that patients taking lovastatin or pravastatin had up to a 70% lower prevalence of AD (Wolozin et al., 2007). Another case-control study reported that patients taking statins (about 60% simvastatin) had also an approximately 70% decreased risk of developing AD (Jick et al., 2000). However, these results might be subject to an indication bias, people who decide to take statins may have a better awareness of their health than those who do not. Therefore, other health-related factors may explain their lowered risk of AD or dementia (Rockwood et al., 2002). In randomized clinical trials, it was found that both treatments of atorvastatin (Feldman et al., 2010) and simvastatin (Sano et al., 2011) showed no significant benefits in patients with mild to moderate AD, and that the use of statins had no association with the onset of AD (Zandi et al., 2005). However, the design of these clinical trials has been criticized due to sampling bias. The population in the clinical trial studies are too homogenous and mainly include white elder males (Zissimopoulos et al., 2017). In the same study, it was found that the reduction in Alzheimer's disease risk by statin treatment varied across different statin molecules, sex, and race/ethnicity and controlled for a prescription bias by looking at only patients who were prescribed statins and segregating them into those who regularly took their medication and those who less frequently took the medication. This most recent study claimed

that additional research efforts are needed and called for cautious attention in designing clinical experiments to test the therapeutic effect of statins. Even though the use of statins in a clinical setting requires more studies, it's still interesting to use statins as a tool to study the influence of cholesterol in AD pathogenesis.

High cholesterol levels in cell culture media was shown to inhibit the secretion of sAPP (Galbete et al., 2000). This reduction in sAPP could be caused by the inhibition of α-secretase or β-secretase activity or both. However, a study showed that the depletion in cholesterol levels increases the non-amyloidogenic α-secretase activity (Kojro et al., 2001). A study found that lovastatin increases glycosylation of mature oligosaccharides in β-secretase, and therefore decreases its activity (Sidera et al., 2005). One study suggested that γ-secretases activity was not affected by Rosuvastatin (Crestini et al., 2011). Thus, the major effect of cholesterol depletion may be the downregulation of β-secretase. In our project, we test whether these findings are reproducible in our model system and attempt to learn more about the mechanism of how atorvastatin affects APP processing.

Recently it was discovered that APP has a flexible transmembrane domain that contains a cholesterol binding site at the β-CTF region (Beel et al., 2010). Through NMR and sequential alanine mutagenesis screening, it was found that both E693 and F691 in the βCTF region are unable to bind to cholesterol when mutated to alanine (Barrett et al., 2012). Many researchers have studied pathogenic mutations on E693. The E693Q mutation (Dutch), located at position 22 in Aβ, may enhance Aβ protofibril formation compared with APP-wild-type (WT) (Kumar-Singh et al., 2002; Nicholson et al., 2012; Knight et al., 2015). The E693K mutation (Italian), also located at position 22 in Aβ, has similar clinical features to E693Q (Nilsberth et al., 2001; Bugiani et al., 2010). The E693G (Arctic) mutation carriers showed decreased Aβ42 and Aβ40

levels in plasma, however it enhances Aβ protofibril formation (Nilsberth et al., 2001). The E693Δ (Osaka) mutation has previously shown to cause dementia by enhancing the formation of synaptotoxic Aβ oligomers, while also causing a marked reduction in Aβ40 and Aβ42 secretion from transfected cells (Tomiyama et al., 2008). However, no studies have been conducted to study whether these mutations also abolish cholesterol binding. Currently it is unknown whether APP-cholesterol binding influences APP processing. Additionally, no studies have yet shown how mutations that abolish APP cholesterol binding such as E693A and F691A influence APP processing. Exploration of the E693A and F691A mutations could provide more insight into the linkage between cholesterol and APP processing.

Using hiPSC-derived NSCs and neurons to study the mechanism of how cholesterol depletion and cholesterol binding site mutations affect APP Processing

Animal models are critical for understanding disease pathogenesis and serve as valuable tools for preclinical testing. However, mouse models with mutations in APP, PSEN 1 and/or PSEN 2 genes do not exhibit all AD pathologic phenotypes at once. (Drummond and Wisniewski, 2017; Jan kowsky et al., 2017). Using human tissues bypasses the limitation of how mouse models do not exhibit all AD pathological features. However, it is difficult to obtain a tissue sample specific to our target mutations and raises challenges to prevent post-mortem protein degradation. We can differentiate human induced pluripotent stem cells (hiPSCs), developed by Takahashi et al 2007, into human neurons and study the living cells in a dish. Using CRISPR/Cas9 gene editing technology, we can induce point mutations in a gene of interest and study the effects of these mutations. CRISPR/Cas9 gene editing functions by delivering the Cas9 nuclease complexed with a synthetic guide RNA (gRNA) into a cell, which

performs an incision. We can use this system to remove and/or add new genes at the desired location (Jinek M et al 2012, Zhang et al., 2014, Jinek et al., 2014). We used CRISPR-CAS9 to induce point mutations in human induced pluripotent stem cells (hiPSCs). Mutations generated include the mutations that abolish APP cholesterol binding, E693A and E693A+F691A. We are also using Atorvastatin to create a cholesterol depletion condition. We hope to learn the mechanisms of how cholesterol depletion and cholesterol binding site mutations alter Aβ production.

RESULTS

Effect of cholesterol binding site mutations on APP processing

Altered cholesterol levels have been implicated in aberrant formation, aggregation, and toxicity, of Aβ-related peptides (Maulik et al., 2013). However, we still lack a complete understanding of how cholesterol can influence AD pathogenesis. We aim to learn more about the effect of cholesterol on APP processing. We obtained hiPSCs and used CRISPR/CAS9 technique to induce E693A single, and E693A+F691A double, mutations. These mutations were previously shown to abolish APP-Cholesterol binding (Barrett et al., 2012). We have obtained two wild-type (WT) cell lines, two E693A single mutation cell lines, and one E693A+F691A double mutation cell line. We then differentiated the wild-types and mutant hiPSCs into neural progenitor cells (NPCs). The NPCs were differentiated to mix-culture neurons(dNPCs) for 3 weeks in 10cm plates prior to protein extraction. We found a decrease in Aβ42 levels and no change in APP FL levels in both E693A single, and E693A + F691A double, mutations (Fig 1A, B, Vanessa Langness Unpublished, van der Kant R et al., 2019). The decrease in Aβ42 observed may indicate that cholesterol binding site mutations affect APP processing to Aβ. Since no changes were detected in APP FL in our mutants, we questioned if the amyloidogenic pathway product, βCTF, was affected by cholesterol binding site mutations. We saw βCTF baseline levels are increased (Fig 2A) and confirmed that APP FL levels remain unchanged (Fig 2B) in cholesterol mutants. Together, these findings indicate that E693A and E693A+F691A APP mutations affect APP processing by increase βCTF and decreasing Aβ42 levels.

Effect of atorvastatin on APP processing in wild-type and cholesterol-binding-site mutants

To study how statins affect APP processing, we aged the dNPCs for 10-14 days after re-plating them in 24 well plates and treated them with 10μm atorvastatin dissolved in dimethyl

sulfoxide (DMSO) for five days. An equivalent volume of DMSO, with no atorvastatin, was used for untreated controls in both wild-types and mutants. Statins inhibit 3-hydroxy-3-methylglutaryl-CoA(HMG-COA) reductase, an enzyme that converts HMG-COA to mevalonic acid, which eventually is converted to cholesterol. Thus, statins decrease intracellular cholesterol levels. Previous studies have found that statins decrease Aβ levels, which led to us hypothesize that statins affect APP processing. Since cholesterol binding site mutations abolish cholesterol binding, we hypothesized that the decreased intracellular cholesterol level caused by atorvastatin does not affect APP processing in our mutants. Therefore, we expect to see little to no changes in APP fragmentation after treating cholesterol binding mutants with atorvastatin. To study how atorvastatin affects APP processing, we treated wild-type cells with atorvastatin and measured APP processing products. When we treated wild-type neurons with atorvastatin, we saw a decrease in Aβ42 and sAPPβ levels and an increase in APP FL levels. When we treated mutants with atorvastatin, we saw the same significant increase in APP FL in E693A single mutant and decrease in sAPPβ in both single and double mutants as atorvastatin-treated wild-types. However, we saw that the reduction of Aβ42 levels was blunted in E693A single mutant compared to its DMSO control. Furthermore, Aβ42 levels were found to be not significantly different in E693A+F691A double mutant compared to its DMSO control (Fig 1D, E, F). To further test the hypothesis, we then asked: does atorvastatin affect β-CTF levels in wild-types and mutants. We are also interested in measuring APP FL in atorvastatin-treated wild-types and mutants. We observed no changes in β-CTF levels in both atorvastatin treated wild-types and cholesterol mutants (Fig 3 A). Although we saw a significant increase in APP FL in both atorvastatin-treated wild-types and E693A single mutants, no significant changes in APP FL was detected in atorvastatin-treated E693A + F691A double mutants (Fig 6D). The differences

observed in APP FL and Aβ 42 in atorvastatin-treated wild-types and mutants led us to suspect that the mutations on the cholesterol binding site are affecting the changes caused by atorvastatin. However, to learn more about the mechanism of how atorvastatin affects APP processing, we asked: does atorvastatin affect β- & γ-secretase levels in wild-types and mutants? To answer the question, we then measured levels of β-secretase(BACE 1), and the y-secretase catalytic subunits PSEN 1, and PSEN 2 in atorvastatin-treated wild-types and mutants. To explain the observation of increased APP FL, no changes in β-CTF, and decreased Aβ42, in both atorvastatin-treated wild-types and mutants, we hypothesized that γ-cleavage and β-cleavage are both decreased in atorvastatin-treated wild-types. The increase in β-CTF resulting from decreased γ-cleavage would be reduced back to control levels with the concurrent decrease in β-CTF resulting from decreased β-cleavage. Since less changes in APP FL and Aβ 42 were observed in atorvastatin-treated mutants, we hypothesized that the decrease in γ-cleavage and β-cleavage was blunted in atorvastatin-treated mutants. We observed no changes in atorvastatin-treated PSEN 2 levels in both atorvastatin-treated and untreated wild-types and cholesterol mutants (Fig 4D). While PSEN 1 showed similar results to PSEN 2, we found that atorvastatin increased PSEN 1 levels in E693A single mutants (Fig 4A). This observation rejects our hypothesis as we expected to see a decrease in γ-secretase subunits in both atorvastatin treated WT and mutants. β-secretase levels were reduced significantly in both atorvastatin-treated wild-types and cholesterol mutants (Fig 4G). Therefore, although the observation of decreased β-secretase levels matched our previous hypothesis that there is a decrease in β-cleavage in both atorvastatin treated wild-types and mutants, we found that β-secretase was decreased to an equal extent in the atorvastatin-treated mutants compared to atorvastatin treated wild-types. Together, these results confirm that atorvastatin affects APP processing, and these effects are reduced in

cholesterol binding mutants. However, the mechanism of how atorvastatin and cholesterol binding site mutations cause these observations require further discussion and exploration.

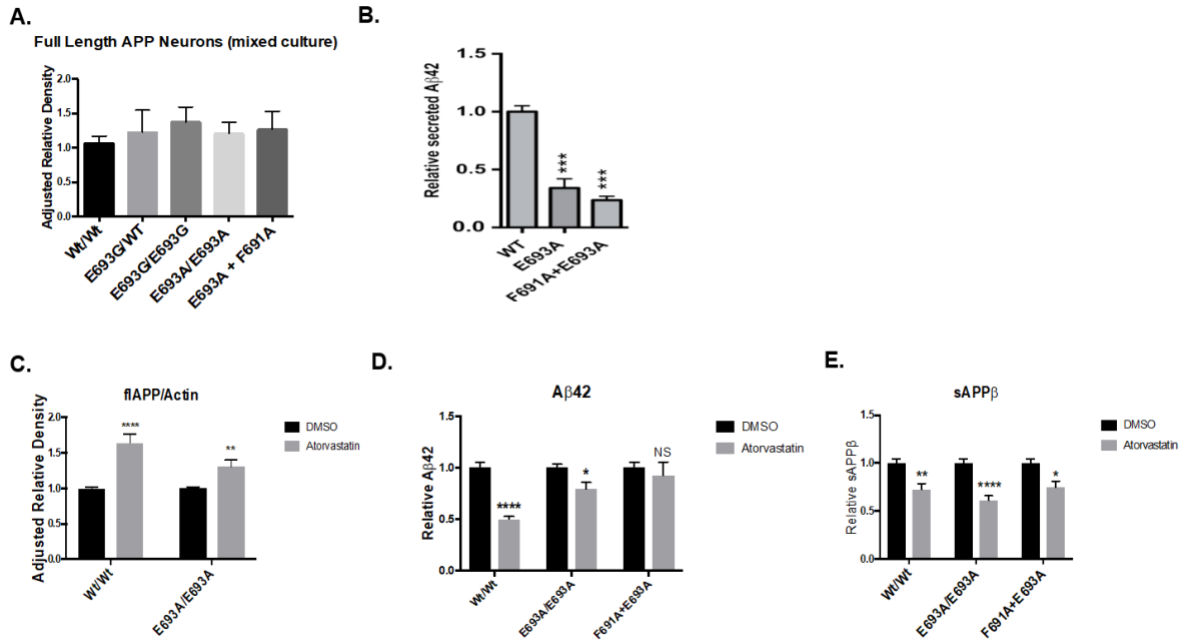

Figure 1. Summary of previous findings. Results of previous studies conducted by van der kant et al 2019 and Langness V, unpublished. Measurement of baseline levels of APP FL(A) and Aβ 42(B) in WTs and cholesterol mutants. Measurement of APP FL(C), Aβ 42(D), and sAPPβ(E) in WTs and cholesterol mutants treated with DMSO control and atorvastatin. Error bars represent SEM from three biological replicates per experiment. Additional experiments were repeated and showed similar results. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001 by One-way ANOVA.

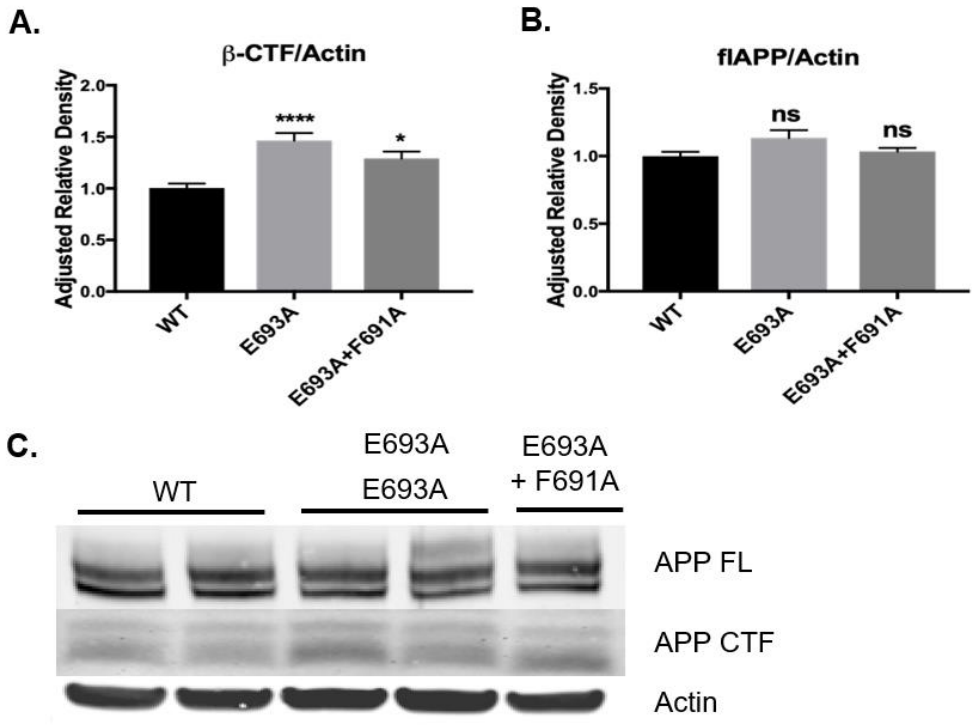

Figure 2. Baseline APP FL and CTF levels in cholesterol mutants and WT. Quantification of β-CTF(A) and APP FL(B) normalized to actin. Image of Western blot (C). Error bars represent SEM from three biological replicates per experiment. Additional experiments were repeated and showed similar results. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001 by One-way ANOVA.

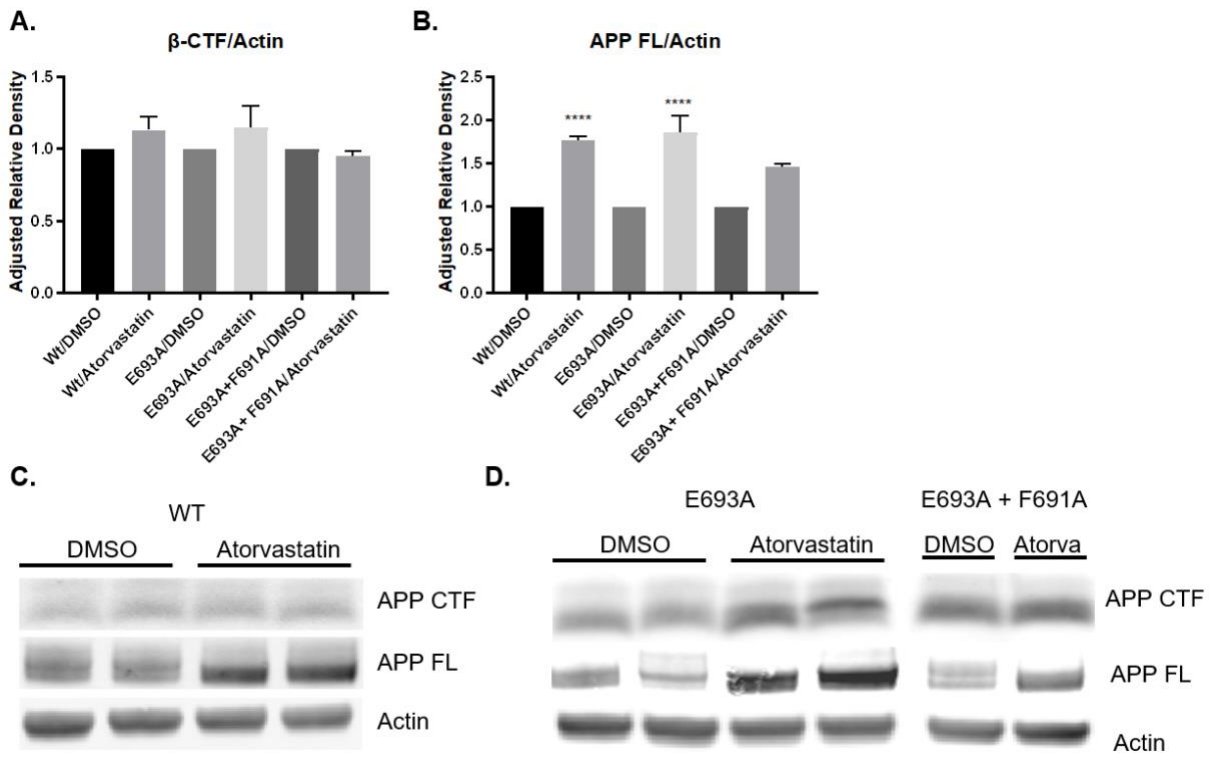

Figure 3. APP CTF and APP FL levels in cholesterol mutants and WT under cholesterol depletion. Quantification of of β-CTF(A) and APP FL(B) levels normalized to Actin in WT and cholesterol mutants when treated with atorvastatin and DMSO, with western blot image (C, D). Error bars represent SEM from three biological replicates per experiment. Additional experiments were repeated and showed similar results. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001 by One-way ANOVA.

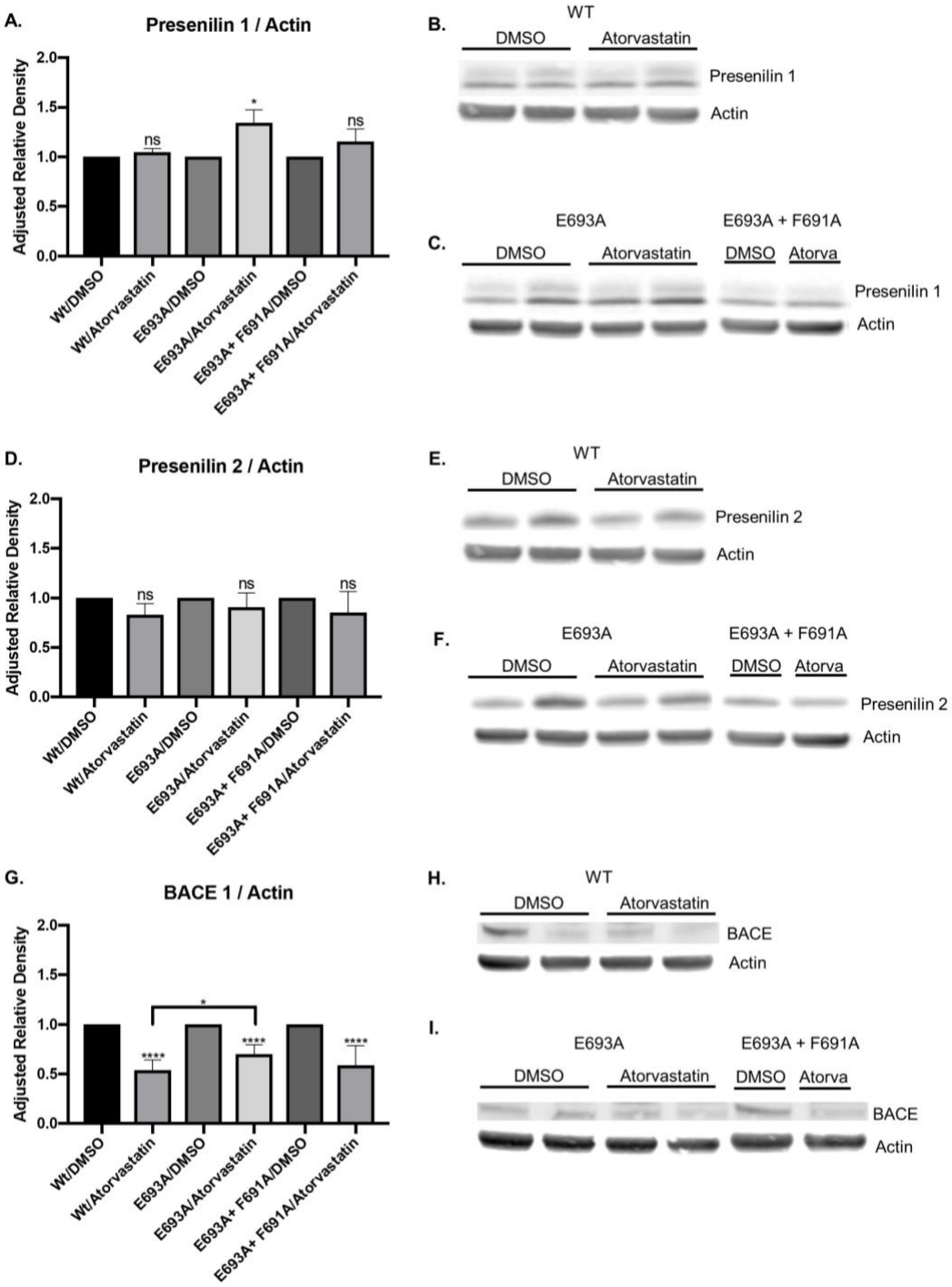

Figure 4. γ -secretase subunits (PSEN 1 & PSEN 2) and β -secretase (BACE 1) levels in cholesterol mutants and WT under cholesterol depletion. Quantification of PSEN 1(A), PSEN 2(B), BACE 1(C), normalized to Actin for WT and cholesterol binding site mutants under atorvastatin and DMSO treatments Western Blot image (B, C, D, F, H, I). Error bars represent SEM from three biological replicates per experiment. Additional experiments were repeated and showed similar results. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001 by One-way ANOVA.

DISCUSSION

Cholesterol binding site mutation affects APP processing

Aβ42 was previously found to accumulate the most in extracellular Aβ plaques in AD patients. Aβ42 was a product resulting from the cleavage interaction between γ secretase and β-CTF, which was produced by β-cleavage of APP FL. Cholesterol was found to be associated with Aβ production, and our studies have shown that cholesterol binding site mutations E693A and E693A+F691A affect APP processing in hiPSC derived mixed-culture human neurons by decreasing Aβ42 levels and increasing β-CTF levels. This finding led us to hypothesize different mechanisms of how these changes can be generated. Currently, there exists no literature that explored the cholesterol binding site mutations and their relation to amyloidogenic APP processing. However, some hypotheses that explain the changes observed include a downregulation in γ-cleavage and an upregulation in β-cleavage. There also exists the possibility that processing changes observed in cholesterol binding mutations can be explained by a combination of both hypotheses. To further test our hypothesis, we will collect baseline levels of β-secretase, PSEN 1 and PSEN 2 to complete the picture of how cholesterol binding site mutations affect APP processing to Aβ.

Although extensive studies have been conducted on APP E693 amino acid mutations, F691 amino acid mutations were rarely explored. Furthermore, no published literature investigated both amyloidogenic APP processing products and secretase enzyme levels in E693 mutations. With the finding that suggests E693A and F691A abolishes cholesterol binding, we believe the study of how these mutations affect APP processing is an important contribution to unravel the linkage between cholesterol and APP with relevance to AD pathogenesis.

Atorvastatin affects APP processing

Next, we explored the effect of atorvastatin on APP processing in wild-type neurons. In the most recent research using simvastatin on blood-brain barrier epithelial cells, simvastatin was found to cause increased APP FL and total secreted Aβ oligomer levels and decreased β-secretase activity levels (Zandl-Lang M et al., 2018). However, the only agreeable change we observed was the increased level of APP FL in atorvastatin-treated wild-type neurons. The differences we see may be attributed to the fact that our study is done in a different cell type (neurons) and the different statin molecules used. Previous research has also claimed that cholesterol depletion decreases β-cleavage (Sidera et al., 2005) and doesn't affect γ-cleavage (Crestini et al., 2011). Based on our observation of decreased Aβ42, increased APP FL, and no changes in β-CTF levels, in atorvastatin-treated wild-types, we initially hypothesized that atorvastatin downregulates both β- and γ- cleavage. Our observation of decreased β-secretase levels supports both our hypothesis and the previous claim made by Sidera et al., 2005. However, our results of seeing no change in both PSEN 1 and PSEN 2 levels after statin treatment supports the findings by Crestini et al., 2011, but not our hypothesis. Nevertheless, also in Crestini et al., 2011, they found that rosuvastatin alters gene expression of the core subunits of γ-secretase complex without affecting its activity. This finding potentially indicates that gene expression might not be directly correlated to protein activity. Thus, our original hypothesis that stated a downregulation in γ-cleavage may still be valid. To further test our hypothesis, additional experiments are needed. Researchers have shown that the γ-secretase complex contains important core components, besides presenilin 1 or 2, that are involved in regulating APP- γ-secretase interaction: Nicastrin, APH-1, and PEN-2 (Carroll et al., 2016; Chow et al., 2010). Therefore, it's essential also to measure the baseline levels of these proteins. On top of

that, to study the interaction level between APP and γ-secretase, co-immunoprecipitation(CO-IP) is an important technique that needs to be developed and optimized. To test our hypothesis that claims a downregulation in both β- and γ- cleavages, we need to perform experiments using CO-IP with APP pull-down and visualization of β-secretase, PSEN 1/ 2, Nicastrin, APH-1, and PEN-2. Since statin is a drug that receives significant attention for its potential clinical relevance in AD, we hope our results will provide a valuable contribution in looking at the complete picture of how atorvastatin affects the pathogenic processing from APP FL to Aβ42.

Effect of Atorvastatin on APP processing is reduced in Cholesterol binding site mutants

Although the majority of changes on APP processing observed in atorvastatin-treated wild-types are also reflected in atorvastatin-treated mutants, we saw a lessened reduction in Aβ42 and lessened increase in APP FL. We initially hypothesized that atorvastatin does not affect mutants as the mutations abolish APP-cholesterol binding. However, our observation partially rejected our hypothesis and indicated that the effect of atorvastatin on APP processing is reduced in mutants. Our new hypothesis includes 1) the reduction of both β- and γ- cleavages caused by atorvastatin is blunted in cholesterol binding mutants and 2) cholesterol binding site mutations are associated with regulatory mechanisms of APP FL and Aβ42 that are independent of APP processing. The mutations could increase APP FL endocytosis (Haass et al., 2012) or inhibit the transportation of APP FL to the membrane (Thinakaran et al., 2008), and could increase Aβ42 endocytosis or inhibit its secretion from the cell. To test the first hypothesis, we need to rely on the CO-IP experiment discussed previously. To test the second hypothesis, we need to develop an optimized co-localization assay to visualize the colocalization of APP FL and Aβ42 with endosomes.

Effect of Atorvastatin on β-secretase expression levels is independent from Cholesterol binding site mutants

Another interesting observation we saw was that β-secretase levels were decreased in both atorvastatin treated wild-types and mutants when comparing to their untreated controls. This observation means cholesterol depletion causes a downregulation in β-secretase protein levels that is independent of cholesterol binding site mutations. We will need to measure the mRNA level of β-secretase to see if the gene transcription rate is altered in cholesterol depletion condition for both wild-type and mutants. We are also interested in measuring any potential increased β-secretase degradation by inhibiting known protein degradation pathways. Our result is interesting and unexpected. Cholesterol depletion was thought to affect APP processing, and naturally, our attention was with the subsequent changes in APP processing products like Aβ and CTFs. However, this finding indicates that the secretase enzyme levels could be regulated by cholesterol levels, which provide a new perspective on cholesterol and APP processing.

Cholesterol regulates APP processing

In the end, we found that cholesterol binding site mutations E693A and E693A+F691A affects APP processing by decreasing Aβ 42 and increasing β-CTF levels. We observed that atorvastatin treatment in wild-type neurons also decreases Aβ42 levels, but caused increased APP FL levels, and no changes in β-CTF levels. We hypothesized that cholesterol depletion caused by atorvastatin downregulates both β- and γ- cleavages. In atorvastatin treated mutants, we observed a lessened decrease in Aβ 42 levels, and a lessened increase in APP FL levels, compared to atorvastatin treated wild-types after normalization to untreated counterparts. Our

hypotheses of this observation include 1) cholesterol binding site mutations inhibit the effect of atorvastatin on APP processing; 2) cholesterol binding mutations affect cholesterol regulated behavior of APP FL and Aβ42 independent from APP processing, such as APP FL clearance/transport and Aβ 42 clearance/secretion. To test these hypotheses, we can use co-immunoprecipitation to pull-down APP and measure secretases protein levels to study the APP-secretase interaction and use Co-localization assay of APP products in different cellular organelles and endocytosed complex to study protein clearance/transport.. Finally, we also observed a decrease in β-secretase levels in both atorvastatin treated wild-types and mutants. This observation suggests cholesterol depletion caused by atorvastatin downregulates β-secretase expression levels independent from cholesterol binding site mutations.

MATERIALS AND METHODS

Cell Lines

All the mutated cells and the baseline wild type controls were made in the same genetic background of hiPSCs derived from Craig Venter's fibroblast cells. Stem cells were differentiated to neural progenitor cells (NPCs), and then differentiated three weeks into a mixed culture of neurons (dNPCs). NPCs from an AD patient with an APP duplication (APP DP) genotype and CRISPR-CAS9 induced APP knock-out neural progenitor cells (APP KO) were used as positive and negative controls.

Cell Culture

All Cultures were incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere. NPCs were grown and maintained in Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12, GlutaMAX(DMEM/F12; Thermo Fisher 10565). The culture media was supplemented with B-27, N-2 (Life Technologies), penicillin-streptomycin (100 U/mL, Life Technologies) and fibroblast growth factor (FGF; EMD Millipore, 20 ng/mL). Culture media was changed every other day. When grown to ~95% confluency, NPCs were dissociated with Accutase (Innovative Cell Technologies) and re-plated onto poly-L-ornithine hydrobromide- (PLO, 20 μg/ml) and laminin- (5 μg/mL) coated 10 cm plates for differentiation, at which point FGF was removed from the media. dNPCs were fed twice a week and differentiate for 21 days. Cells were then to be extracted, or to be placed in protein crosslinker(DSG) treatment, or for another re-plating process if used for atorvastatin treatment experiment. These dNPCs were dissociated using Accutase and Accumax 1:1 mixture (Innovative Cell Technologies) and then passed through a 100 μm filter before plating onto PLO/laminin-coated 24 wells plates in NSC media containing 0.5 mM dbCAMP (Sigma), 20 ng/μl brain-derived neurotrophic factor (BDNF; Peprotech), and 20 ng/μl glial cell line-derived neurotrophic factor (GDNF; Peprotech), and laminin (5%) for aging process. dNPCs were counted with filtered Trypan Blue Solution, 0.4% (Gibco), and $2x10^6$ cells were plated on each well. Aging

dNPCs were fed twice a week with media counting dbCAMP, BDNF, GDNF, and laminin. The aging process was allowed for two weeks prior to atorvastatin treatment. One-Half media change was performed with atorvastatin in 10 μm suspended in aging media. Equal amount of DMSO-media solution was used as a vehicle control for studies for atorvastatin.

Protein Crosslinker Treatment

dNPCs were washed with Phosphate-buffered saline(PBS). Disuccinimidyl Glutarate (DSG, Thermo Scientific™ 20593) solution was prepared at 25mM in Dimethyl sulfoxide(DMSO) with 1x Hault protease Inhibitor cocktail (Thermo Scientific™ 78430), EDTA free. dNPCs were then treated with DSG solution at final concentration of 1mM diluted in PBS for one hours at 37°C prior to extraction.

Protein Extraction

Sample cells were washed with cold Phosphate-buffered saline (PBS). Cells were then extracted with 1x RIPA buffer(10X RIPA, Water, Hault phosphatase inhibitor, Halt protease inhibitor, EDTA) and lysed with 27.5 Gage needling. Supernatant from centrifugation at 4°C, 20,000 rcf for 20 minutes were collected and total protein concentrations were determined through BCA assay(Pierce 23225).

Western Blot

Lysates were diluted to 30ug prior to adding 6x Sample Buffer(6X loading dye, 20mM beta mercaptoethanol) follow by heating at 100°C for 10minutes. Samples were cooled to room temperature prior loading on a 1.5mm 4-12 bis tris gel. Samples were ran with 1x MES buffer at room temperature, 110 V for 1 hour and 30 minutes, and transferred with 1x transfer buffer(10X

Tris-Glycine transfer Buffer, 1:5 Methanol) at 4°C, 0.3A for 45 minutes. Transferred membranes were washed once with TBST prior to blocking with Odyssey Blocking Buffer (PBS) for one hour at room temperature. Blocked membranes were then washed once with TBST prior to be placed in primary antibody for overnight incubation at 4°C. Incubated membranes were then washed with TBST, placed in secondary antibody and shaken for one hour at room temperature. Three washes with TBST and one wash with PBS were performed prior to developing using Odyssey® CLx Imaging System. Antibody anti-APP, C-Terminal 751-770 (Millipore 171610) was used to visualize APP CTF. Antibody Anti- β-secretase, EPR3956 (ab108394). Antibody Anti-Presenilin 1, C-Terminal 263-378 (MAB5232). Antibody Anti-Presenilin 2, C-Terminal 300-400 EP1515Y (ab51249). Antibody Human/Mouse β-secretase Ectodomain Antibody (MAB931). Secondary Antibody rabbit TrueBlot®: anti-rabbit IgG HRP mouse monoclonal (eB182).

Statistics

For each figure, statistics were performed by using either a one-way ANOVA with Dunnet correction for multiple comparisons, or by two-tailed Student's t-test using GraphPad Prism 6. The data were presented as the mean ± SEM.

Table 1. Summary of results

All data collected with sample size of 3. Atorvastatin treated sample data for APP FL and βCTF have a sample size of 6. * Previous data collected by Vanessa Langness, unpublished; van der Kant et al., 2019. - Data not collected

	E693A vs. WT	**E693A + F691A vs. WT**	**DMSO treated WT vs. Atorvastatin treated WT**	**DMSO treated E693A vs. Atorvastatin treated E693A**	**DMSO treated E693A + F691A vs. Atorvastatin treated E693A + F691A**
βCTF	Increased $P \leq 0.0001$	Increased $P \leq 0.05$	No Change	No Change	No Change
APP FL	No Change*	No Change*	Increased $P \leq 0.0001$	Increased $P \leq 0.0001$	No Change
PSEN 1	-	-	No Change	Increased $P \leq 0.0001$	No Change
PSEN 2	-	-	No Change	No Change	No Change
BACE 1	-	-	Decreased $P \leq 0.0001$	Decreased $P \leq 0.0001$	Decreased $P \leq 0.0001$

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