LAKE FOREST COLLEGE

Senior Thesis

The Alternative Splicing of ApoER2 in Alzheimer's Disease

by

Daniella Brutman

December 7, 2011

The report of the investigation undertaken as a Senior Thesis, to carry two courses of credit in the Departments of Biology and Neuroscience

Michael T. Orr Krebs Provost and Dean of the Faculty	Shubhik K. DebBurman, Chairperson
	Dawn Abt-Perkins
	Robert Glassman
	Michelle Hastings Rosalind Franklin University of Medicine & Science

Abstract

Over five million adults over the age of 65 currently suffer from Alzheimer's disease (AD), a debilitating disease with no cure. Early diagnosis of AD allows for an increased ability to manage the disease, but diagnosis is limited by a lack of reliable AD biomarkers. While there is no singular cause, research has linked an isoform of ApoE to early onset AD, but recently more attention has been placed upon one of ApoE's receptors, ApoER2. Like most genes, ApoER2 undergoes multiple alternative splicing events to create distinct mRNA isoforms. One Splicing event, exon 18 inclusion vs. skipping, creates two isoforms with opposing functions in reelin signaling, synaptic plasticity, and memory functions. We hypothesize that ApoER2 alternative splicing correlates with AD progression. Using cellular and animal models, and human samples, we found a disease state association between increasing AD severity and increased ApoER2 exon 18 skipping. We conclude that ApoER2 may play a role in regulating a pathway that is deregulated in AD.

Acknowledgements

This thesis would not have been possible without the support of a handful of individuals. First, I would like to thank Dr. Michelle Hastings for the opportunity to work in her lab; her mentorship throughout this process proved indispensable. I would also like to acknowledge Dr. Shubhik DebBurman for his never-ending support and guidance throughout not only this process but my entire experience at Lake Forest College. Thank you for constantly pushing me to achieve more than I knew I was capable of. Additionally, I would like to thank Professor Dawn Abt-Perkins and Dr. Robert Glassman for working with me on a rushed time table to make this thesis possible. My research would not have been possible without those who previously worked on this project, including: Paige, Angela, etc. The support and guidance of Fran Jodelka, Mallory Havens, Alicia Case, Anthony Hinrich, Cidi Wee, Ashley Reich, Kate McCaffrey, and Alejandra Luna also allowed me to learn more than I could have hoped, and their ability to put up with my never-ending questions still astounds me.

Lastly, I would like to thank those that have supported me throughout not only this endeavor by my entire life as well, my family. Without their support I would never have been able to get to this point in my life. I also need to thank my peers in the biology and neuroscience departments for helping me throughout the past few years and my friends in Delta Gamma Fraternity for their constant affection and ability to make sure I kept my sanity as I pushed myself to complete this capstone in my education.

Table of Contents **Page**

List of Figures **Page**

List of Tables **Page**

List of Abbreviations

HD: Huntington's disease

PD: Parkinson's disease

AD: Alzheimer's disease

Aβ: amyloid beta

APP: amyloid precursor protein

NFT: neurofibrillary tangles

SNP: single nucleotide polymorphism

ApoE: apoliprotein E

LDLR: low-density lipoprotein receptors

LTP: long term potentiation

RR: reelin repeats

Dab1: Disabled-1

ApoER2 or LRP8: apoliprotein E receptor 2

Introduction

Alzheimer's Disease

Alois Alzheimer first described Alzheimer's disease (AD) in 1907 after observing tangles and plaques in the brain of a patient that had previously suffered from cognitive impairments and confusion (Alzheimer, 1907). Now, AD is quickly becoming one of the most prevalent diseases of the twenty-first century. AD is the most common form of dementia, accounting for 60 to 80% of all cases. In America alone, 5.4 million Americans suffered from AD in 2011 (CDC, 2011; Heber, et al., 2003). Currently, one in eight people over the age of 65 and one in two people over the age of 85 suffer from AD, and these numbers are expected to rise drastically as the baby boomer generation continues to age.

The brains of AD patients often display shrunken gyri, widened sulci, and an atrophied hippocampus and amygdala (Yarri & Corey-Bloom, 2007; Khairallah & Kassem, 2011). These larger deviations are brought on by several molecular changes, including the extracellular accumulation of amyloid beta (Aβ) and intracellular accumulation of hyper-phosphorylated tau (figure 1A; Rebeck, et al., 2006). Together these changes lead to the selective cell death of neurons, initially in the hippocampus, that spreads to other brain regions as the disease progresses (Coleman & Flood, 1987). The presence of Aβ plaques and tau neurofibrillary tangles (NFT), the hallmarks of AD, are correlated to declines in 6 possible domains of cognitive function: global cognition, working memory, semantic memory, episodic memory, perceptual speed, and

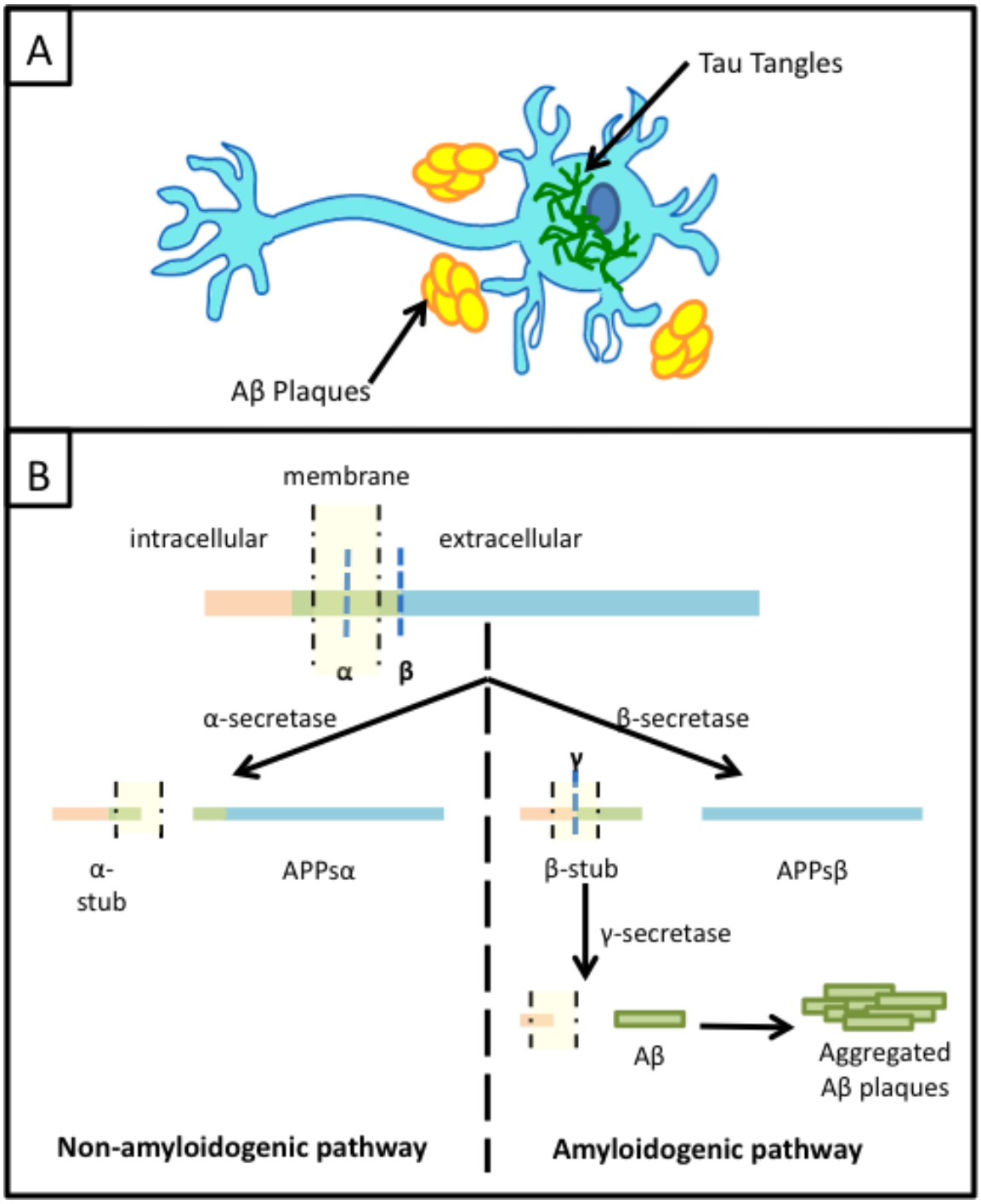


Figure 1. APP cleavage is disrupted in AD to form amyloidogenic Aβ. (A) Extracellular Aβ plaques and intracellular tau NFTs characterize AD. **(B)** APP has multiple cleavage site that determine if the non-amyloidogenic or amyloidogenic pathway is used. In healthy neurons, APP is cleaved via the non-amyloidogenic pathway by α-secretase to create APPsα and an α-stub that remains in the intracellular side of the cell attached to the membrane. In individuals with AD, APP is first cleaved by β-secretase

and creates a shorter APPsβ and a β-stub that protrudes into the extracellular space. The β-stub is then cleaved again by γ-secretase to create a neurotoxic form of Aβ that is able to self-associate and form aggregated Aβ plaques, the product of the amyloidogenic pathway.

visuospatial ability (CDC, 2011; Klunk & Abraham, 1988; Selkoe, 1986).

Despite the amount of research on the underlying pathology of AD, no cure or effective treatment exists. Currently five drugs on the market can slow the progression of the disease for up to one year, including: tacrine, donepezil, rivastigmine, and galantamine (Doody, et al., 2001). Yet, these drugs are only effective for 50% of AD patients (CDC, 2011). AD's prevalence has spurred research leading to almost 100 treatments in human trials that are also attempting to slow the progression of the disease (CDC, 2011). However, to increase the success of most AD therapies currently in trials, early detection and treatment are necessary. Current research hypothesizes that molecular changes take 10 years to culminate in the clinical signs of AD, a time period in which disease diagnosis may be critical to treatment success (Galindo, et al., 2010). Diagnosing or tracking the progression of AD during this critical time requires a biomarker, which have yet to be discovered.

While predisposition to AD can be determined through testing for known genetic risk factors, there is currently no method to determine onset or progression of the disease. If doctors were able to determine the progression of the disease in its asymptomatic stage, treatment efficacy could increase drastically. However, we have no effective biomarker to catch AD at an early enough stage for treatment to be optimal. Additionally, finding this precursory change may be important in finding a target for potential AD therapies.

ApoE: a genetic risk factor for AD

Only one known genetic risk factor for Late Onset AD (LOAD) has been associated with disease state: Apolipoprotein E (ApoE). ApoE, a 299 amino acid long

protein, is a signaling molecule that is important for cholesterol transport (Ma, et al., 1994). A correlation between ApoE and CSF tau levels leads scientists to believe that ApoE may play a role in tau tangle formation through cholesterol metabolism. ApoE occurs in three different alleles defined by different single nucleotide polymorphisms (SNPs). Each of the three ApoE isoforms— ε2, ε3, and ε4— codes for a different protein. ApoE-ε3, the most common isoform, is a neutral ApoE isoform, while ε2 and ε4 alter an individual's chance of developing AD. ApoE-ε2 is associated with delayed onset and decreased odds of developing AD (Corder, et al., 1993). On the other hand, ApoE-ε4 is associated with an increased risk for the development of AD, and earlier AD onset (Corder, et al., 1993; Rebeck, et al., 1993). ApoE-ε4 accounts for almost 50% of the risk of developing LOAD (Lahiri, et al., 2004; Rocci, et al., 2003).

While, the three isoforms only differ by two amino acids each, they have much larger associations with AD risk (Rebeck, et al., 2006). The different activities of the ApoE isoforms are linked to the molecular changes that occur in AD, including the extracellular accumulation of amyloid beta (Aβ) and plaque formation. Aβ plaques are a cleavage product of the amyloid precursor protein (APP). APP, found in most neurons, can be cleaved through two different pathways, the non-amyloidogenic and amyloidogenic pathways, due to the multiple locations at which it can be cleaved (Figure 1B). In healthy neurons, the non-amyloidogenic pathway is more common and occurs when APP is cleaved by α-secretase to create APPsα and an α-stub that remains in the intracellular side of the cell attached to the membrane (Esch, et al., 1990). In AD patients, instead of α-secretase cleavage, APP undergoes cleavage by β-secretase via the amyloidogenic pathway. This creates a shorter APPsβ and a β-stub that protrudes into the

extracellular space; the β-stub is then cleaved again by γ-secretase to create a neurotoxic form of Aβ that is able to self-associate and form aggregated Aβ plaques, the product of the amyloidogenic pathway (Vassar, et al., 1999). Aβ, a protein that occurs naturally in all human brains in two forms, is then secreted into the extracellular neuronal space (Walsh & Selkoe, 2007; Finder & Glockshuber, 2007). The 40-peptide long form of Aβ is most common in healthy brains, and a longer 42-peptide long Aβ ($A\beta_{42}$) is the form found most often in aggregated extracellular Aβ plaques (Kar, et al., 2004). While the presence of $A\beta_{42}$ alone does not cause the neurodegeneration created by Aβ-aggregation, its self-association forms the hallmark Aβ plaques of AD that are looked to as the main inhibitor of neuron-to-neuron communication at the synapse (Pleckaityte, 2010; Herrup, 2010).

ApoE regulates Aβ aggregation by forming a stable complex with Aβ, modulating Aβ induced neuroinflammation, and specifically associating with $A\beta_{42}$ (Strittmatter, et al., 1993; Ma, et al., 1994; Guo, et al., 2004). ApoE-ε2, the ApoE isoform that is associated with a decreased risk of AD, is the most effective isoform for the promotion of synaptic plasticity and protects neurons against non-fibrillar Aβ induced death. On the other hand, ApoE-ε4, the isoform that confers an increased risk for the development of AD, is associated with increased levels of $A\beta_{42}$ (Corder, et al., 1993; Rebeck, et al., 1993).

Since Aβ binds to ApoE and is found in most AD Aβ deposits, it has been suggested that ApoE receptors may act as disposal mechanism by transporting Aβ across the blood brain barrier or moving Aβ to astrocytes for later degradation (Zlokovic, 2004; Trommer, et al., Dis). While each ApoE isoform may indicate a different risk for AD

development, they all bind to low-density lipoprotein receptors (LDLRs) to mediate signaling. ApoE receptors have been a focus of AD research, as it is considered a promising therapeutic intervention point that could be targeted to control Aβ plaque formation. One LDLR receptor, APOER2, was originally identified as an ApoE receptor based on its ability to bind to and mediate ApoE signaling. However, a major role for ApoER2 in AD has emerged by virtue of its interaction with the signaling molecule reelin, which plays an important role in cognitive function, and more specifically synaptic transmission and plasticity in the hippocampus (Weeber, et al., 2002; Beffert, et al., 2005).

ApoER2 and the reelin pathway

Reelin is highly conserved between most vertebrates, and at normal concentrations, reelin and Aβ antagonize each other at the synapse (Tissir & Goffinet, 2003; Durakoglugil, et al., 2009). In the synapse, Aβ leads to the dephosphorylation of the NMDA receptor (NMDAR) NR2B subunit; this induces the endocytosis of the NMDAR—suppressing synaptic function. NMDAR endocytosis decreases synaptic plasticity by creating long term potentiation (LTP) deficits such as those seen in AD that inhibit learning and memory. Reelin signaling through ApoER2 opposes this by promoting the phosphorylation of NR2B, therefore, inhibiting NMDAR endocytosis and antagonizing Aβ at the synapse (Figure 2; Durakoglugil, et al., 2009).

Reelin is a large extracellular protein secreted by Cajal-Retzius (CR) cells on the surface of developing brains throughout migration (Sinagra, et al., 2005; Tissir &

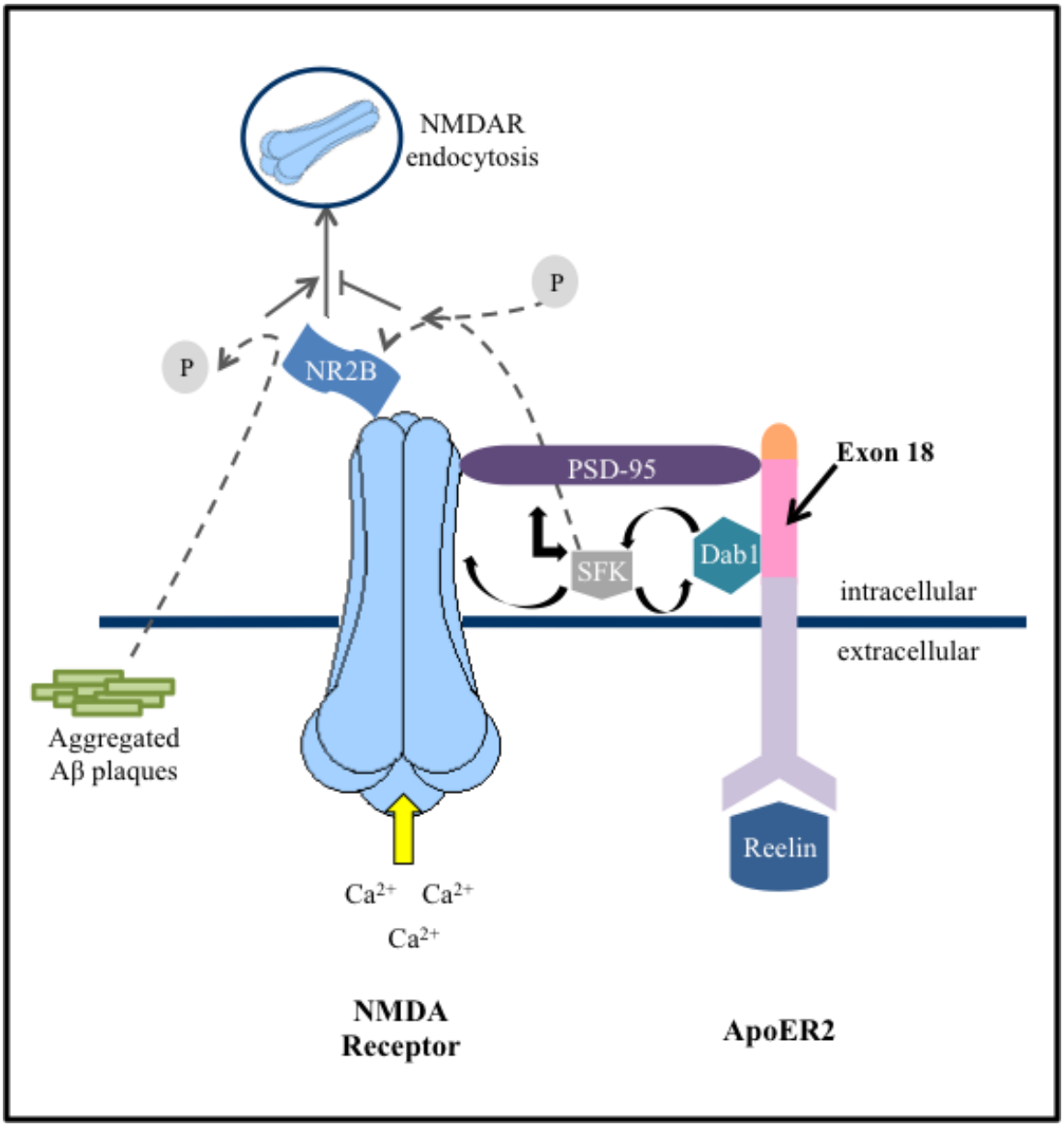


Figure 2. Reelin antagonizes Aβ in the synapse. (A) Reelin binds to ApoER2's extracellular domain which activates Dab1 phosphorylation. This in turn recruits and activates the Fyn/Src SFK cascade which continues to phosphorylate Dab1. Through the attachment of ApoER2 to NMDARs via PSD-95 on the NPxY of exon 18, this signaling pathway is able to induce the influx of calcium via the NMDAR. Aβ in the synapse leads to the dephosphorylation of the NMDAR NR2B subunit and induces the endocytosis of the NMDAR, which suppresses its synaptic function. Conversely, through ApoER2, the reelin pathway promotes the phosphorylation of both NR2B and NR2A NMDAR subunits. Thus, reelin signaling may inhibit NMDAR endocytosis and acts as an antagonist for Aβ at the synapse, which may be important for normal synaptic functioning. (Adapted from Durakoglugil et al. 2009)

Goffinet, 2003) and GABAergic neurons in the hippocampus for LTP and neuronal alignment in adults (Beffert, et al., 2005; Hiesberger, 1999; Trommsdorff, 1999; Weeber, et al., 2002). Mice lacking reelin suffer from LTP defects, amoung other AD characteristics (Durakoglugil, et al., 2009). Furthermore, in excitatory synapses with decreased function due to AB plaques, reelin signaling can restore synaptic plasticity (Durakoglugil, et al., 2009). Inhibition of the reelin pathway by blocking reelin or its receptors, specifically those from the LDLR family such as ApoER2 (Yasui, et al., 2007), results in reduced dendritic growth and LTP in cultured hippocampi neurons.

ApoER2 is a 60 kb genomic region on chromosome 1p34 (Ma, et al., 2002). Commonly referred to as LRP8, ApoER2 is necessary for the proper lamination of the cortex and migration of later generated neurons (Duit, et al., 2010; Hack, et al., 2007). Binding of reelin to ApoER2 is necessary for dendritic spine formation in hippocampal neurons and the reelin pathway (Niu, et al., 2008). Reelin binds to ApoER2 and activates Disabled-1 (Dab1), a cystolic adaptor protein that receives reelin signals, through tyrosine phosphorylation. This reelin signaling cascade depends upon a Dab1 interaction with ApoER2's conserved NPxY domain, which is coded for by exon 18 of the APOER2 gene (Bock & Herz, 2003; Arnaud, et al., 2003; Hiesberger, et al., 1999). Exon 18 encoded amino acids are also necessary for the interaction with c-Jun amino (N)-terminal kinase (JNK) interacting proteins and other scaffolding proteins such as PSD-95 (Stockinger, 2000). This interaction with PSD-95 is particularly important because of PSD-95's direct interaction with NMDARs (Beffert, et al., 2005). PSD-95, which is believed to be important for synapse maturation, co-precipitates with exon 19 in mouse neurons, showing that they have a direct interaction in this model as well (Gong & Lippa,

2010; Hoe, et al., 2006).

Differential expression of exon 18-containing isoforms of APOER2

Though, exon 18 of APOER2 encodes a protein domain that is critical for Reelin signaling, not all forms of APOER2 have this domain. ApoER2 can code for multiple protein isoforms by regulating splicing of its pre-messenger RNA (mRNA). Genes have both introns (non-coding regions) and exons (coding regions), and splicing is the process by which introns are removed and exons are ligated together to form an mRNA that encodes the contiguous coding sequence that is then translated into a protein. Alternative splicing is a process in which a single pre-mRNA can yield multiple mRNAs and ultimately protein isoforms, by including or excluding (skipping) particular exon sequences. Splicing and regulation of splicing occurs via a large multiprotein complex called the spliceosome, which recognizes various splicing sequences in the pre-mRNA. These splicing sequences include the actual splice junction sequences, which define the borders between exons and introns, as well as multiple regulatory elements.

Regulatory elements can fit in one of four categories: exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs). ESEs promote inclusion through the recruitment of SR splicing factors, while ESSs inhibit inclusion by blocking the recognition of exon splice sites (Graveley, 2000); healthy cells have a balance between both ESEs and ESSs (Zhu, et al., 2001). Similarly, ISSs inhibit exon splicing by recruiting repressors, while ISEs act antagonistically near the same region (Wagner & Garcia-Blanco, 2001). The regulation

of splicing via alternative splicing pathways is an important mechanism by which to regulate protein expression and produce proteins with altered functions. In the case of APOER2 exon 18, alternative splicing may be critical in regulating reelin signaling and deregulation of this splicing event could contribute to cognitive decline in AD (Figure 3A).

In healthy individuals, both forms of the mRNA and protein are found in both the young and old. A balance between the form active in reelin signaling (+exon 18 isoform) and the inactive form (-exon 18 isoform) may be in important in the regulation of Reelin signaling. Isoforms lacking exon 18 are dominant negative inhibitors of the reelin signaling pathway and lead to early defects in LTP (Durakoglugil, et al., 2009). By indirectly modulating NMDAR conductance, ApoER2 affects intracellular calcium levels and therefore LTP (Rebeck, et al., 1993). The inclusion of this exon is also essential for the coupling of the activated Dab1-SFK complex and ApoER2 receptor to NMDARs in the postsynaptic density (PSD) of the synapse (Beffert, et al., 2006; Chen, et al., 2005). In AD patients, a drastic rearrangement of the PSD, specifically in frontal cortical tissues, is seen; this altered synaptic structure may be an underlying cause for decreased synaptic plasticity and defects in synaptic functions (Hering & Sheng, 2001). Additionally, ApoER2 interacts with APP to stabilize it and decrease $A\beta_{42}$ production by increasing α-cleavage (Rebeck, et al., 1993).

Studies in mice have provided a strong link between APOER2 alternative splicing and cognitive function. Similar to human exon 18, in mice, ApoER2 exon 19 can either be skipped or included. ApoER2 knockout mice develop problematic neuronal organization in the hippocampus, which leads to AD-like behaviors such as deficits in

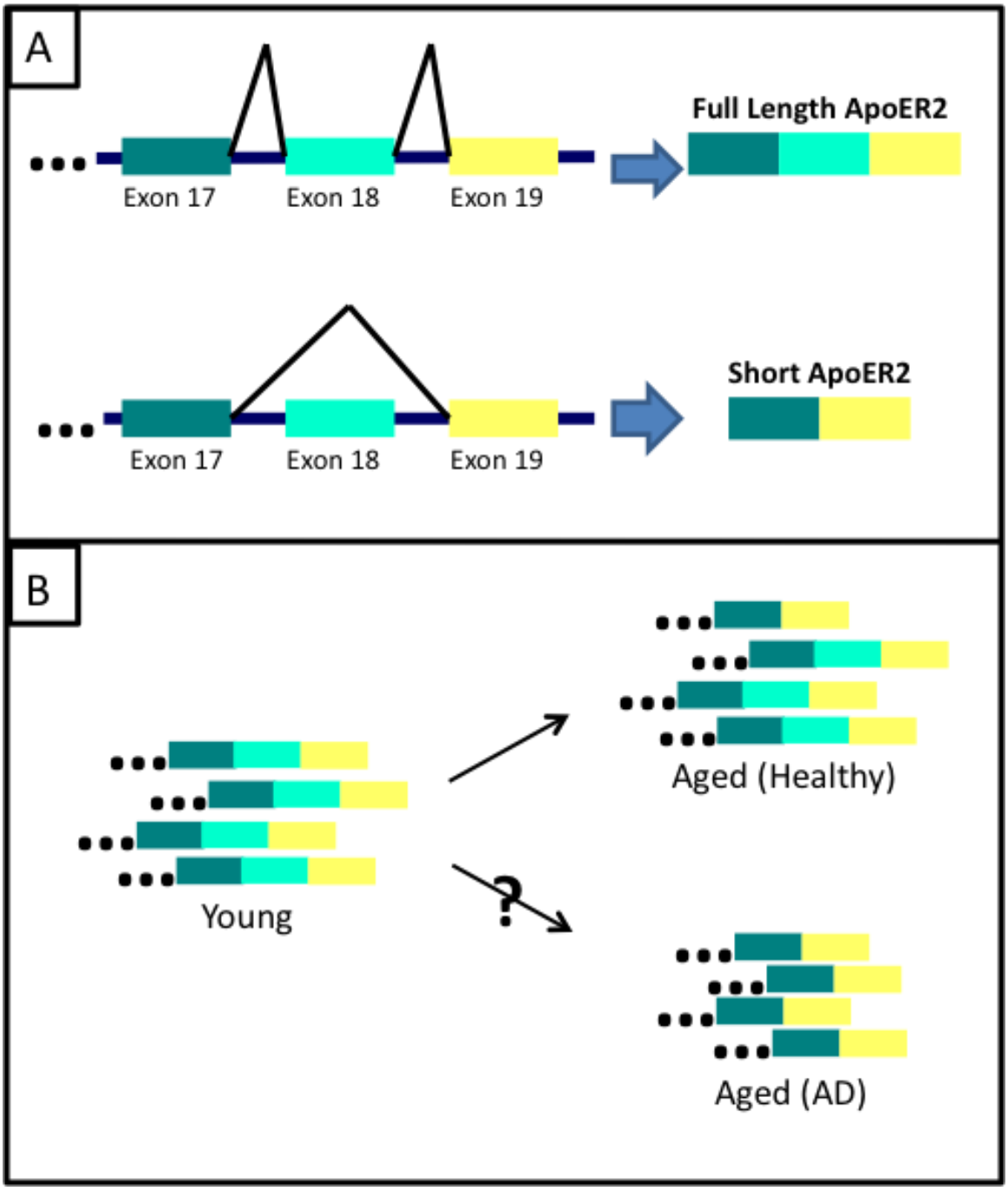


Figure 3. ApoER2 alternative splicing creates multiple isoforms that may change in population in AD. (A) The full length form of ApoER2, which includes exon 18, is found in most individuals. Alternative splicing can also be used to create the shorter isoform of ApoER2 that skips exon 18. The short isoform is found in most Aβ plaques. **(B)** ApoER2 undergoes multiple splicing events that may change over time. Exon 18 is generally excised in young and aged healthy individuals, but we hypothesize that exon 18 inclusion decreases in AD patients as disease severity increases.

memory and learning tasks (Ma, et al., 2002; Beffert, et al., 2005; Halushka, et al., 1999). Beffert et al. (2005) created two different transgenic mice. Both were null for the APOER2 gene (Apoer2$^{-/-}$) but one was transgenic for an APOER2 cDNA that included exon 19 and the other one was transgenic for a cDNA lacking exon 19. Even though ApoER2 is necessary during development, they found that exon 19 is not required for neuronal lamination in the neocortex or hippocampus. However, Apoer2$^{-/-}$ mice rescued with a transgene expressing the Apoer2 isoform lacking exon 19 developed LTP deficits. Furthermore, they discovered that exon 19 is necessary for reelin signaling enhanced LTP and NMDAR-dependent synaptic responses (Beffert, et al., 2005).

The evidence that regulation of exon 18/19 alternative splicing is important for cognitive function in humans and mice leads to the hypothesis of this project: that a decrease in APOER2 exon 18 inclusion is associated with AD in humans (Figure 3B).

Preliminary Research

Addressing this hypothesis, previous research in our lab found that exon 18 skipping increased as AD state progressed. A decrease in the amount of long form ApoER2, including exon18, compared to short form expressed was seen as AD state progressed (Figure 4A). An ANOVA was run by David Bennett and Bryan James and analyzed with a linear regression. Samples were adjusted for sex, level of education and age of death. The average exon 18 inclusion (± standard error of the mean) was 51.99% ± 2.438 in non-cognitive impaired (NCI), 49.18% ± 2.190 in mild cognitive impaired (MCI) samples, and 38.53% ± 3.454 in AD samples (Figure 4B). There was a significant

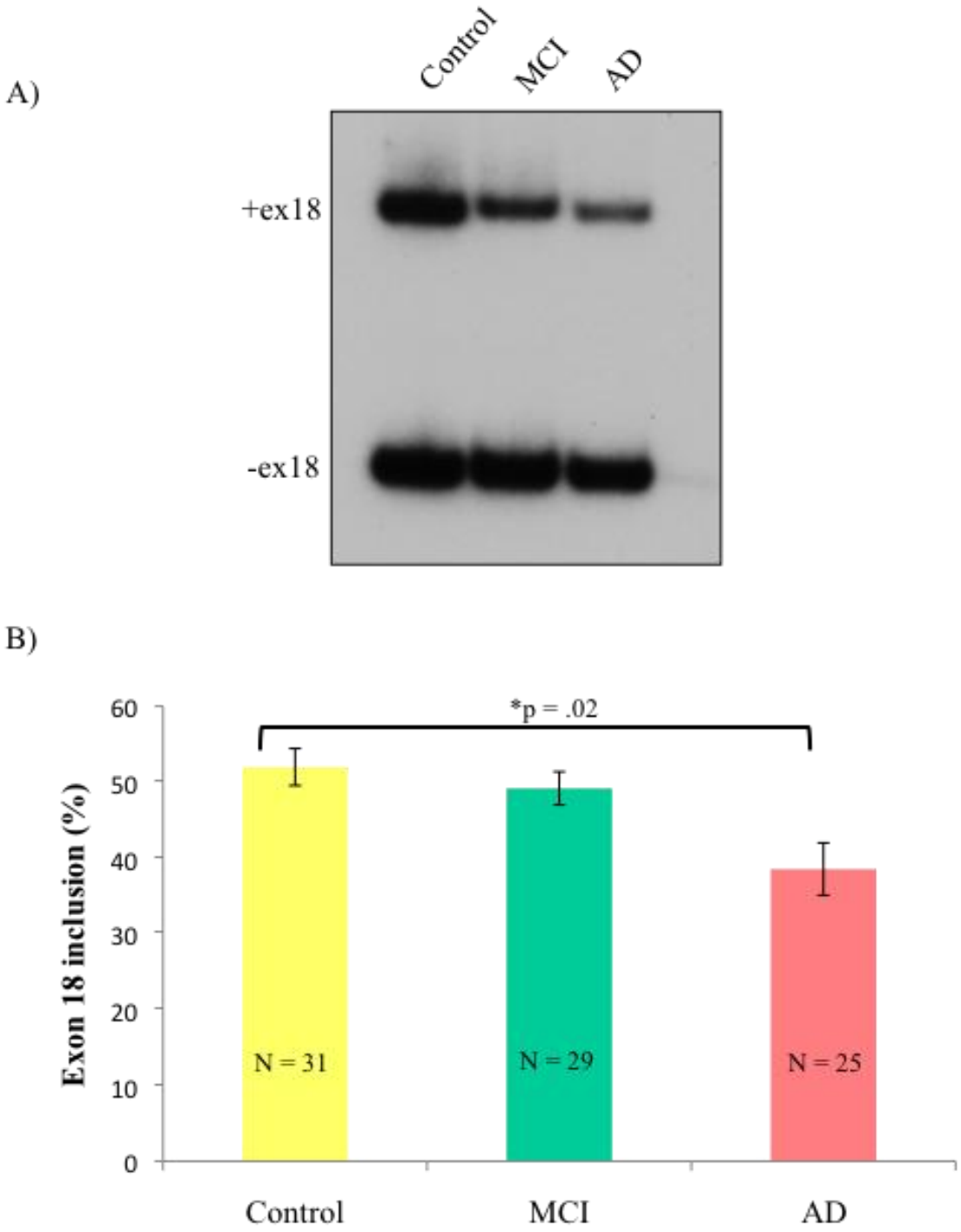


Figure 4. Exon 18 skipping in human mid-temporal brain samples increases in AD. (A) RT PCR of 85 human mid-temporal brain samples; run on a 6% acrylamide gel with ^{32}P-dCTP. Upper bands include exon 18, and lower bands skip the alternatively spliced exon. **(B)** Average percent inclusion for control, MCI, and AD conditions. Significant increase in exon 18 skipping in AD patients compared to control patients (*p< 0.05) also correlated with all six cognitive domains: global cognition (est= 0.019, SE= 0.005, p=

0.0002), working memory (est= 0.012, SE= 0.005, p= 0.0196), semantic memory (est= 0.019, SE= 0.005, p= 0.0001), episodic memory (est= 0.023, SE= 0.008, p= 0.0033), perceptual memory (est= 0.017, SE= 0.006, p= 0.0098), and visuospatial memory (est= 0.018, SE= 0.006, p= 0.0056). Exon 18 inclusion displayed the most robust correlation with semantic memory, but there was no genome wide significance because $p > 5 \times 10^{-8}$.

decrease in exon 18 inclusion in AD samples compared to NCI samples (F=6.7, p=.02).

Exon 18 inclusion was also tested against AD and its 6 cognitive domains to test for a correlation. Exon 18 inclusion correlated with AD state (est= -0.065, SE= 0.021, p= 0.0017). It also correlated with all six cognitive domains: global cognition (est= 0.019, SE= 0.005, p= 0.0002), working memory (est= 0.012, SE= 0.005, p= 0.0196), semantic memory (est= 0.019, SE= 0.005, p= 0.0001), episodic memory (est= 0.023, SE= 0.008, p= 0.0033), perceptual memory (est= 0.017, SE= 0.006, p= 0.0098), and visuospatial memory (est= 0.018, SE= 0.006, p= 0.0056). Exon 18 inclusion displayed the most robust correlation with semantic memory, but there was no genome wide significance because $p > 5 \times 10^{-8}$.

My project

Gap of knowledge and purpose

Aβ-plaques have been shown to cause neurotoxicity at the synapse through NMDA receptors (Matos, et al. 2008). Reelin's ability to antagonize β-amyloid at the synapse leads to a model in whichApoER2, reelin, and Aβ work together, activate the NMDA receptor, and modulate neurotransmission (Durakoglugil, et al. , 2009). However, the mechanism behind this relationship remains unknown.

Specifically, the purpose of this project is to both define the relationship between the alternative splicing of ApoER2 and understand how it can be manipulated. The overall implications of this research could lead to an AD biomarker that could be used for earlier diagnosis in the disease, a possible target for AD therapeutic screening and

development, and a better understanding of an underlying mechanism of AD.

Hypothesis

We hypothesized that ApoER2 exon 18 inclusion correlates with AD progression. Thus, as AD state progresses, ApoER2 exon 18 inclusion decreases regardless of changing neuronal populations (Figure 5).

Aim 1: ApoER2 splicing in human samples

To first test if exon 18 inclusion correlates with AD progression, our lab previously studied 85 post mortem brain samples from Rush Alzheimer's Disease Center's religious order study. We found that as the disease progressed from control to AD, exon 18 inclusion decreased. There was a correlation between exon 18 inclusion and each of the AD cognitive domains. Additionally, these results showed no interactions with ApoE-ε4 status, showing that the change in exon 18 splicing was not an artifact. Thus, in this study we wanted to use quantitative PCR (qPCR) to normalize the data and ensure that we were measuring a change in splicing rather than an increase neuronal death and the proportion of glial cells. We found no correlation between relative RNA abundance and exon 18 inclusion, supporting our hypothesis that we were measuring a change in splicing rather than a change in neuronal populations.

Aim 2: Measuring ApoER2 splicing in different brain sections

Many AD findings have been discovered using one of several mouse models that have mutations in either APP, PS-1, PS-2, or a combination of the three. An AD animal

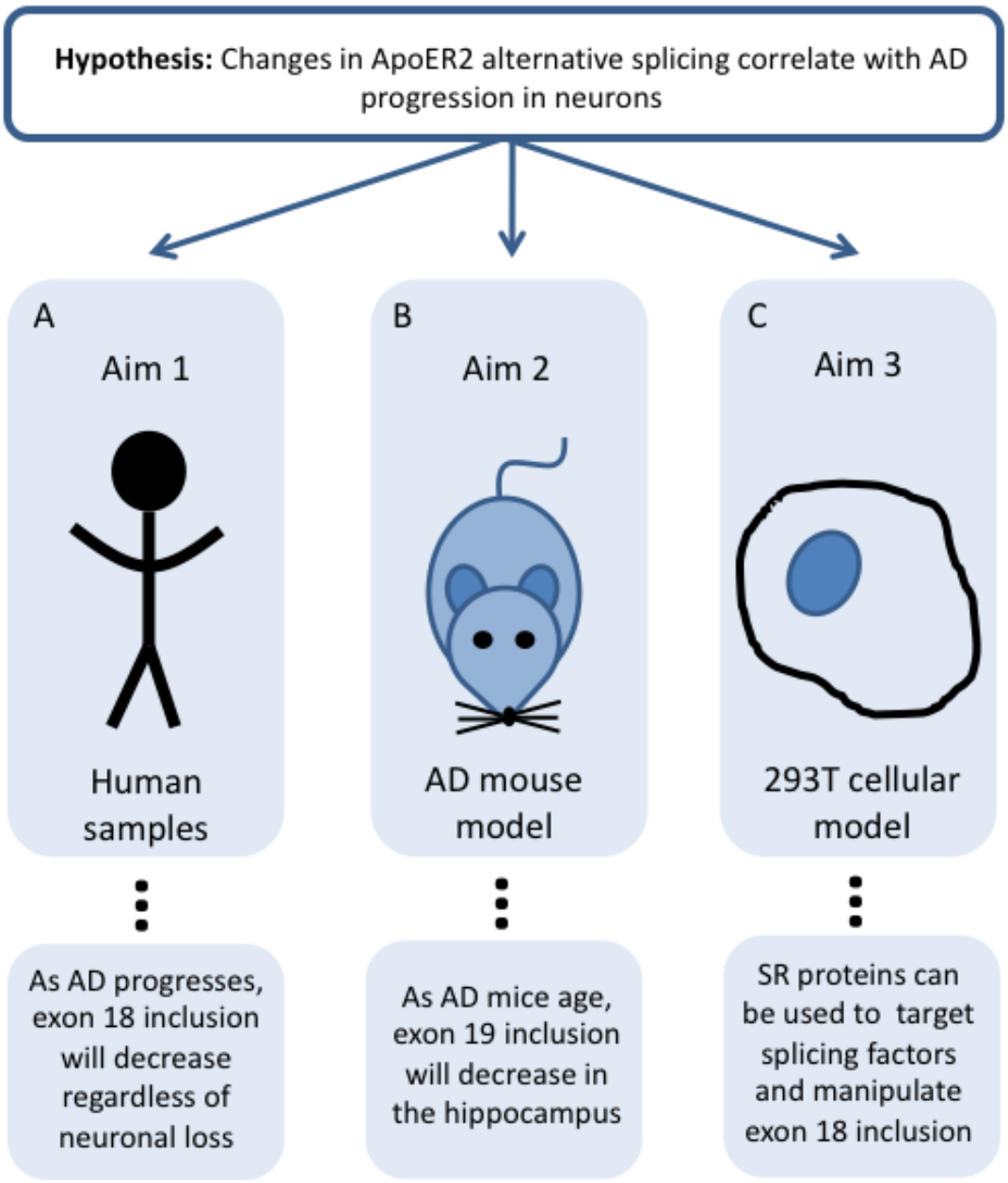


Figure 5. My project hypothesis and aims. We hypothesized that changes in ApoER2 alternative splicing correlate with AD disease state progression independent of neuronal cell death. **(A)** Aim 1 attempts to further preliminary data from out lab by showing that the as AD progresses in human mid-temporal brain samples, exon 18 inclusion will decrease in neurons. **(B)** Once the presence of the alternative splicing change is supported, we will test an AD mouse model. We hypothesized that as AD mice age, exon 19 inclusion will decrease in the hippocampus but remain unchanged in cortex and cerebellum. **(C)** Finally, once the splicing change is seen in 2 models, aim 3 will use a cell model to knockout or over express SR protein sequences in an attempt to increase exon 18 inclusion.

model must exhibit cognitive impairment and age-related neuropathology, and while there are multiple AD mouse models currently being studied, we chose TAS mice due to their availability and demonstration of age-related LTP deficits (Howlett, et al., 2004). APP transgenic mice develop elevated Aβ levels at a young age and extracellular Aβ plaques in the hippocampus and cortex at about 9-12 months (Holcomb, et al., 1998; Games, et al., 1995). Mice with PS-1 mutations present with a subtle elevation of $A\beta_{42}$ levels, but no abnormal pathology (Holcomb, et al., 1998). The most accurate AD mouse model has both APP and PS-1 mutations. These mice develop Aβ plaques at about 6 months of age (Pugh, et al., 2007). Additionally, APP/PS1 transgenic mice develop a 41% increase in $A\beta_{42}$ in the hippocampus and cortex (Holcomb, et al., 1998). Overall, the APP/PS1 transgenic mouse model, sometimes referred to as the TAS model, is the most accurate depiction of AD and therefore used in this study to apply our findings in human AD samples to another model.

Aside from testing for a change in ApoER2 alternative splicing in another model, we also wanted to test where in the brain ApoER2 alternative splicing correlated to AD state. Mice were sacrificed at either 1-2 months or 5-7 months. After sacrifice, their brains were dissected, and we extracted tissue from the hippocampus, cerebellum and cerebral cortex. We used reverse transcription (RT) and PCR techniques to measure exon 19 splicing in the three brain sections. There was no significant change in exon 19 inclusion between age groups and disease state for the cortex and cerebellum, but there was a significant decrease in exon 19 inclusion in the aged TAS hippocampus samples compared to the young TAS mice.

Aim 3: Manipulating ApoER2 splicing

After finding a correlation between exon 18 inclusion and AD progression, we next investigated whether we could increase exon 18 inclusion by targeting different splicing factors. For this, we knocked-out or overexpressed splicing factors (SR proteins) in cultured cells. A panel of over a dozen splicing factors was assessed to test whether any readily available splicing factor we possessed would change exon 18 inclusion. After transfection with various siRNAs targeted to specific splicing factors for knock-down, the cells were collected and measured for exon 18 inclusion. We found that exon 18 inclusion increased when SRSF1 or SRSF7 was knocked-out, suggesting, that both SR proteins may be potential therapeutic targets of interest.

Methods

qPCR

We obtained 85 NCI, MCI, and AD human mid-temporal brain tissue samples from Rush Alzheimer's Disease Center's religious order study, which was part of the Alzheimer's Disease Genetic Consortium. Previous studies in our lab measured a change in ApoER2 exon 18 alternative splicing in AD patients. To ensure the data was measuring a splicing change rather than a decrease in neurons and increase in glial cells, I normalized the relative RNA abundance to a marker for myelin basic protein (MBP) (Grear et al., 2009). Thus if the decrease in neuronal population is not causing the measured change in splicing, we expected to see no correlation between exon 18 splicing and MBP.

Actin and MBP taqMan gene expression assays were used in separate trials. I loaded the taqMan mix and human mid-temporal brain cDNA into 96 well plates (20 µL per well) and ran them on the Applied Biosystems 7500 Real-Time PCR System. Standard curves were created based on selected wells and report settings. The thermal cycler profile consisted of three stages: 50°C for 2:00 minutes (1 cycle), 90°C for 10:00 minutes (1 cycle), and 95°C for 00:15 minutes and 60°C for 1:00 minute (40 cycles).

Animals

We obtained mice from Dr. Robert Marr's lab. The Rosalind Franklin University's Institutional Animal Care and Use Committee approved all experimental procedures. A total of 32 mice were used and sample demographics can be seen in appendix A. Twenty-five mice were collected in the lab previously; I added 7 mice to

increase the sample size. I sacrificed mice by asphyxiation using a halothane chamber or CO_2 before breaking the neck to ensure mortality. Tissue collection was performed in ice cold PBS. I collected hippocampus, cortex, and cerebellum samples from both hemispheres, immediately placed in liquid nitrogen, and then placed samples in the -80°C freezer until RNA collection at a later date.

RNA Isolation

For animal tissues, I removed the brain samples from the -80°C freezer and placed them on dry ice. I added 1 mL Ambion TRIzol Reagent to each sample (300µL was added to the hippocampus samples) and homogenized using a 2-speed 1000 Omni power homogenizer. The samples were left in the TRIzol reagent for 10 minutes. For cells, I added 1 mL TRIzol to each well outside of the hood. I transferred the TRIzol and cells to a new tube after the 10 minute incubation period.

Following incubation, 200 µL chloroform per mL TRIzol was added before shaking vigorously for 15 seconds, incubating at room temperature for 2 minutes, and spinning at 12,000 xg at 4°C for 15 minutes. Then, I transferred the supernatant to a new tube to which 500µL isopropanol per 1 mL TRIzol was added. Following this, the samples were spun down at 12,000 xg at 4°C for 10 minutes. If the pellet was difficult to see, I added glycoblue and spun the sample again. The supernatant was then removed, and 1 mL 75% ethanol per 1 mL TRIzol was added to the pellet. Next, I spun samples down at 7,600 xg at 4°C for 5 minutes. I then removed the supernatant and left the pellet to dry for 5 minutes. Then, I added 50 µL nanopure dH_2O, briefly vortexed the solution, and left it in a warm water bath for 2 minutes to dilute the RNA pellet. A biophotometer

was used to determine RNA concentrations.

Cell culture and transfection

293t cells were grown in HyClone DMEM/High Glucose media supplemented with 10% FBS. They were split into 6-well plates, with concentrations of 5 x 10^5 cells per well. Cells were incubated overnight at 37°C and 5% CO_2. Transfections were performed when cells were over 90% confluence. Transfections solutions consisting of 5 µL siRNA and 250 µL GIBCO opti-mem were given 5 minutes to mix before the addition of a solution of 10 µL Invitrogen lipofectamine 2000 Reagent and 250 µL GIBCO opti-mem. They were then left at room temperature for 20 minutes before being added to the plates. Transfected cells were then incubated for 48 hours; media was changed 24 hours after transfection. After incubation, RNA was collected using the TRIzol protocol.

RT and PCR

Human and Cell samples

RT was performed using Go Script Reverse Transcriptase from Invitrogen. 1µg RNA was brought up to 10µL with dH_2O. 1 µL oligo (dt) was added to each tube before incubating samples at 70°C for 5 minutes and chilling on ice for 5 minutes. The 5x Reaction Buffer, 25mM $MgCl_2$, 10mM dNTPs, and Go Script RT were added to each sample in a 4:3:1:1 ratio. Samples were incubated at room temperature for 5 minutes, 42°C for 1 hour, and then 70°C for 15 minutes. The resulting cDNA was then used for PCR.

For radioactive PCR, a 12.5: .1: 9.4: 1: 1 ratio was used for the addition of GoTaq, ^{32}P-dCTP, dH_2O, and the two primers respectively. Primer #90, APOER2ex18F (5'-TGGTGATAG CCCTCCTGTG-3'), and primer #91, APOER2ex20R (5'-TGCATGGGACTGAATTCC-3') were used. The PCR was run for 35 cycles at an annealing temperature of 58°C, and the samples were run on a 6% native acrylamide gel for 2:30 at constant 220 volts.

Mouse samples

RT was performed using SuperScript III Reverse Transcriptase from Invitrogen. A 1:1 ratio mix of oligo (dt) and 10µm dNTP was added to enough dH_2O and RNA to create a 13 µL solution with 1µg RNA. The mixture was then heated at 65°C for 5 minutes and chilled on ice for 1 minute. A master mix consisting of a 4:1:1 ratio of 5x first strand buffer, .1M DTT and Superscript III RT was added before incubation at 50°C for one hour, and heating at 70°C for 15 minutes. The resulting cDNA was then used for PCR.

The same radioactive PCR mix was created with different primers. Primer #378, musAPOERex18F (5'-TGGTAATAGCCCTGCTATG-3'), and primer #379, musAPOERex20R (5'-TGCATAGGACTGAACTCC-3') were used. The PCR was run for 35 cycles at an annealing temperature of 58°C. The same machine and gel protocols were used for mouse and human samples.

Quantification and Statistical Analysis

Percent inclusion was calculated using the following formula: % inclusion = exon18in/ (exon18in + exon 18out) x 100. Relative RNA abundance was calculated using

CT-values and formulas found in appendix B. Statistical significance was calculated in excel using two-tailed student t-test.

Results

Experimental design

We hypothesized that as AD state progresses, ApoER2 exon 18 inclusion decreases in neurons. To test our hypothesis, we used human, mouse and cellular samples. The present study included 85 human samples (31 control, 29 MCI, and 25 AD). Preliminary research in our lab showed that these samples displayed decreased exon 18 inclusion as AD state progressed. To make sure that this change was not an artifact due to changing neuronal and glial cell populations, we normalized the RNA abundance and tested for a correlation between exon 18 inclusion and glial cell population through measuring the presence of a glial cell component, myelin basic protein (MBP). After assuring that the change in exon 18 splicing was not an artifact, we used a TAS AD mice to test for the splicing in another model; 32 mice (5 young control, 7 old control, 11 young AD, and 9 old AD) were used in this study. Using the secondary model, we tested the hippocampus, cerebellum and cortex for a change in exon 19 inclusion as both control and AD mice aged. After seeing the change in ApoER2 alternative splicing in both models, we turned to an *in vitro* cellular model to test whether splicing factors can be over or under expressed to increase or decrease exon 18 inclusion. SR protein sequences that can be targeted to change exon 18 inclusion may be potential targets of interest for genetic therapy.

ApoER2 exon 18 inclusion correlates with AD in human samples

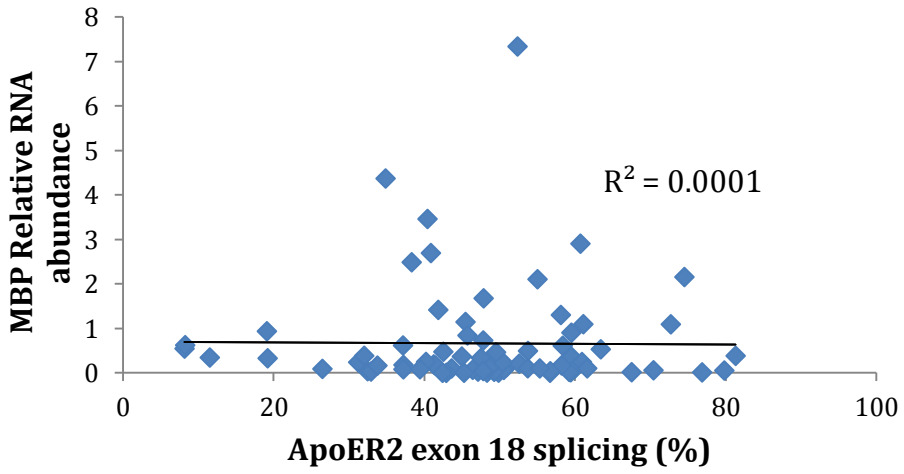
Previous research in our lab showed a decrease in ApoER2 exon 18 inclusion in AD human samples compared to healthy controls. Yet, it was not clear if this change is a

result of increasing neuronal death and thus a relative increase in glial cell populations. Since glial cells may naturally have a different form of ApoER2 splicing with decreased exon 18 inclusion, we wanted to make sure we were measuring a change in exon 18 splicing in neurons. Using MBP as a marker for glial cells, relative MBP abundance was correlated to ApoER2 exon 18 percent inclusion. A qPCR using MBP and actin taqman probes was used to normalize RNA abundance in control (NCI), MCI, and AD human mid-temporal brain samples (Grear, et al., 2009). After normalization, there was no correlation between MBP relative RNA abundance and ApoER2 exon 18 percent inclusion (R^2= 0.0001). The absence of a correlation between glial cell population and ApoER2 exon 18 inclusion shows that the change in ApoER2 exon 18 inclusion previously measured occurs in our cells of interest, neurons (Figure 6A). MBP relative RNA abundance for both MCI and AD conditions was then normalized to control levels to test for a change in cell populations between conditions (Figure 7B). There was no significant difference in MBP relative RNA abundance between MCI and control conditions (p =0.6589) or AD and control conditions (p= 0.4474). Together, these findings support our hypothesis that the decrease in ApoER2 exon 18 inclusion shown in human AD samples occurs in mid-temporal neurons.

Hippocampal ApoER2 exon 19 inclusion decreases with age in AD mice

After finding support for our hypothesis in human mid-temporal brain samples, we chose to measure ApoER2 exon 19 splicing in an animal model, TAS AD mice. This model system provides benefits over human samples because unlike human samples that may be affected by various extraneous variables, these mice are subject to much more

A)



B)

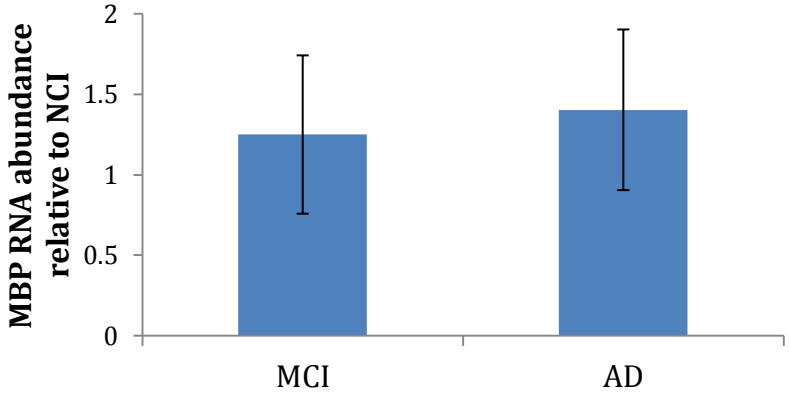


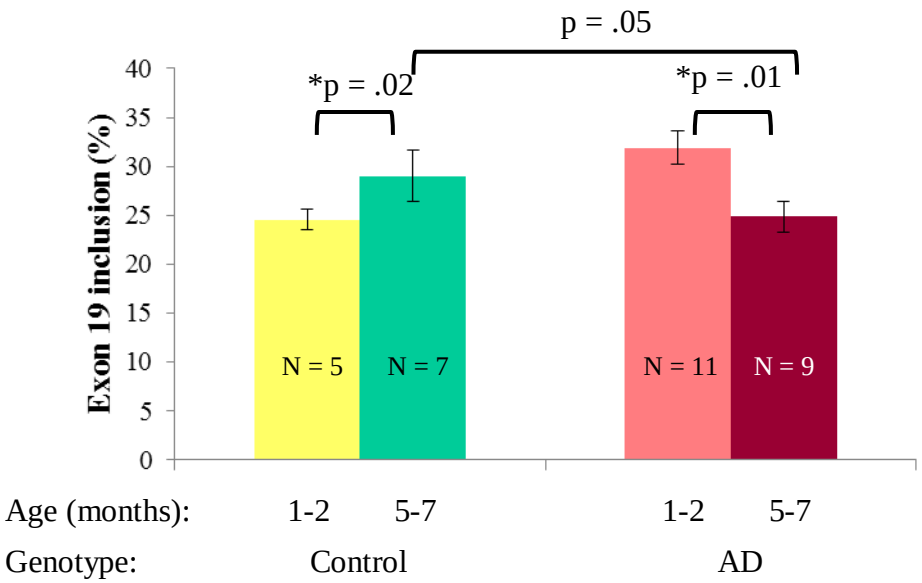
Figure 6. Percent ApoER2 exon 18 inclusion and RNA abundance are not correlated. (A) qPCR with MBP taqman probes was used to normalize RNA abundance to actin in human mid-temporal brain samples. No Correlation was seen between relative RNA abundance and ApoER2 exon 18 percent inclusion (R^2= 0.001). **(B)** MCI and AD mid-temporal brain sample relative RNA quantity was normalized to NCI samples. No significant difference was seen between MCI and NCI conditions ($p>0.05$) or AD and NCI conditions ($p>0.05$).

control. They are raised in the same environment, treated identically, and only differ in their phenotypes. TAS mice, which have APP and PS-1 mutations, are the most severe AD mouse model available, and they develop Aβ plaques near 6 months of age (Pugh, et al., 2007). Both control and TAS mice were collected at a young age of 1-2 months before plaque development, and after plaque presence is expected at 5-7 months. I tested the hippocampus, cerebellum, and cortex for splicing changes. Of the 32 mice, 25 were previously analyzed; thus, I increased the sample size by 7 mice. The population consisted of 20 males and 12 females that were split into the following groups: 5 control 1-2 month mice, 7 control 5-7 month mice, 11 AD 1-2 month mice, and 9 AD 5-7 month mice.

In the hippocampus, AD mice showed a significant average decrease in inclusion from 31.88% ± 1.698 at 1-2 months to 24.84% ± 1.519 at 5-7 months, a decrease of 22.07% ($p= 0.0106$). In opposition, there was a significant average increase in exon 19 inclusion from 24.56% ± 1.096 at 1-2 months to 29.00% ± 2.646 at 5-7 months for control mice, an increase of 18.08%, in the hippocampus ($p= 0.0225$; Figure 7A/B). Overall, there was a percent of 40.87% between the two conditions. Hippocampal samples also showed a significant difference between control and AD mice at 1-2 months ($p = 0.0408$) and at 5-7 months ($p= 0.0479$).

In the cerebellum, no significant change in ApoER2 exon 19 inclusion was observed (Figure 8A). Yet, there was a non-significant trend of decreasing inclusion in aging AD mice, similar to that in the hippocampus ($p= 0.2283$). Additionally, there was no difference in exon 19 splicing in the cortex between control and AD mice (Figure 8B). Since AD symptoms initially develop in the hippocampus, the greatest splicing change

A)



B)

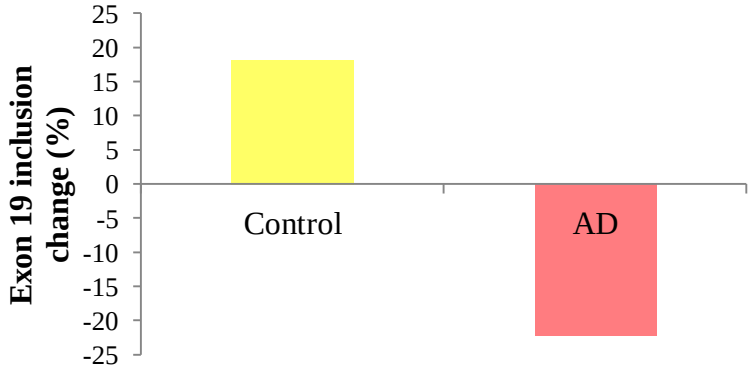


Figure 7. Exon 19 splicing in transgenic AD mouse hippocampus samples. (A) Average percent exon 19 inclusion of 4 conditions shows opposing trends for control and AD mice collected at young (1-2 months) and old (5-7 months) ages. Exon 19 inclusion increased with age in control mice (p= 0.02) and decreased in AD mice (p= 0.01). **(B)** Percent change between young (1-2 months) and old (5-7 months) control and AD mice. Control mice showed an increase of 18.08% and AD mice showed a decrease of 22.07%.

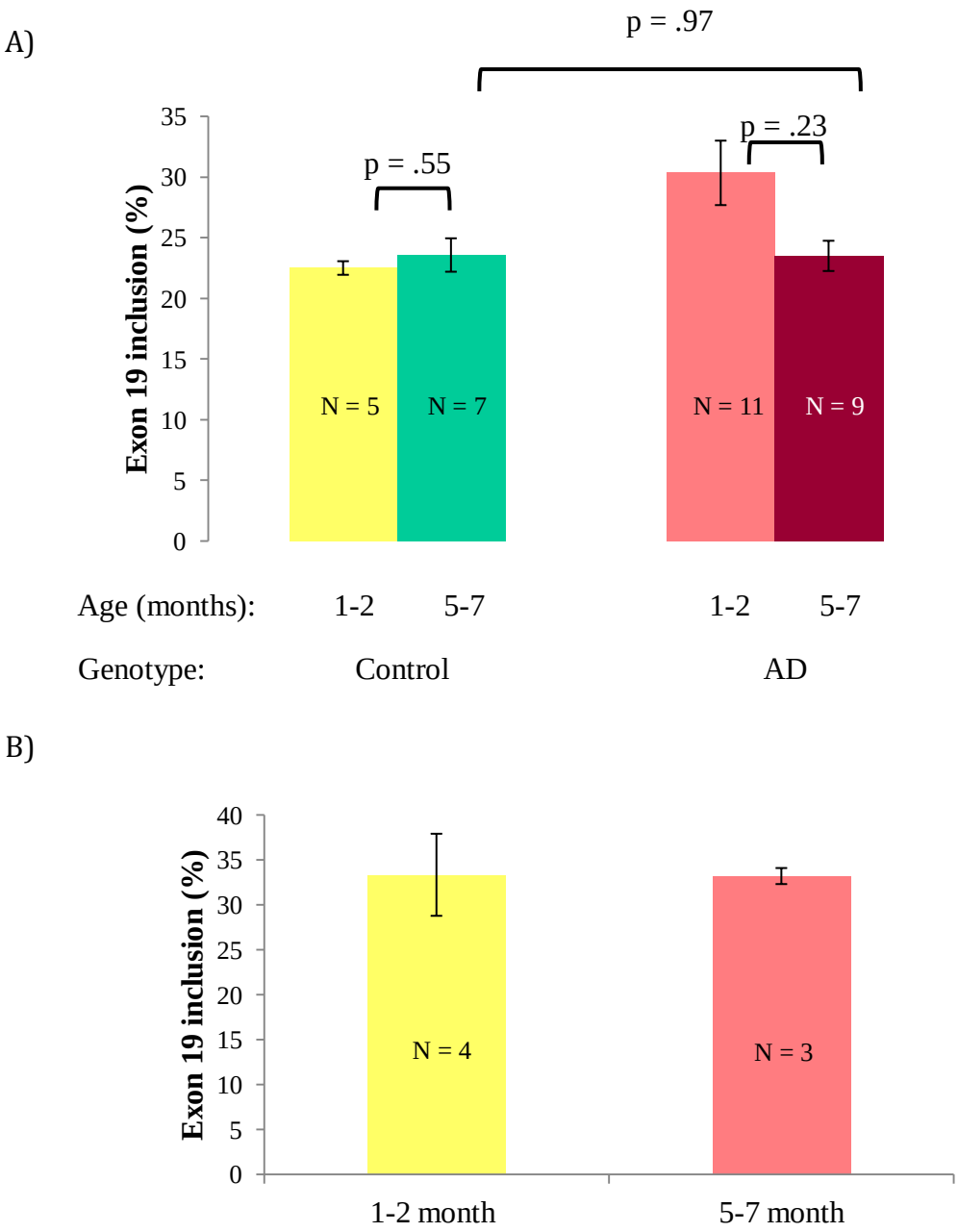


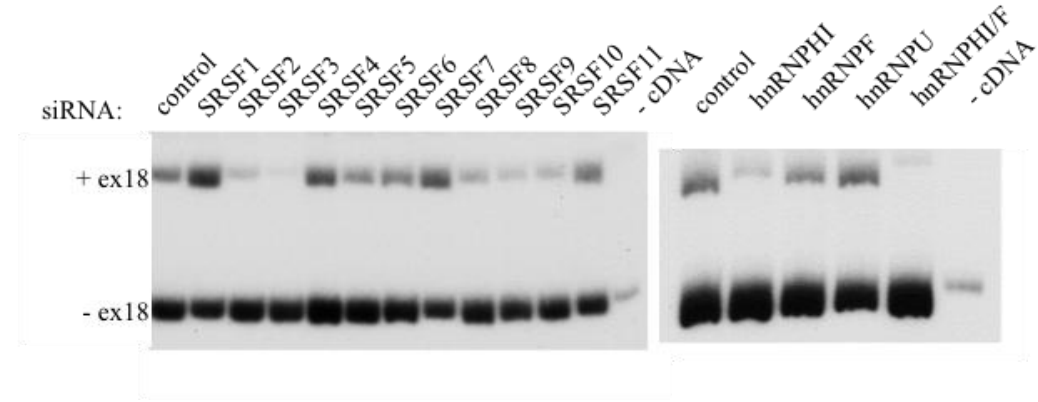
Figure 8. ApoER2 exon 19 splicing in mouse cerebellum and cortex shows no significant change. (A) Average percent exon 19 inclusion of the four conditions. An increasing trend in control mice and decreasing trend in AD mice are seen from young to old mice, but no significant difference change in exon 19 splicing was found between any states ($p>0.05$). **(B)** No change is seen in alternative splicing of exon 19 in AD mouse cortex samples ($p>0.05$)

was expected there. Overall, findings in this mouse model supported our hypothesis that a change in ApoER2 exon 19 splicing is also present in a mouse model.

Targeting splicing factors increases exon 18 inclusion

After finding associations of ApoER2 alternative splicing and AD in humans and mice, we chose an *in vitro* cellular model to manipulate a panel of known splicing factors in an attempt to promote or inhibit exon 18 inclusion and identify regulatory elements that control splicing. If a sequence or splicing factor can regulate exon 18 inclusion, it may serve as a potential therapeutic target. Using siRNAs and sterile tissue culture technique we knocked out SR protein sequences in HEK 293T cells. After extracting RNA, I ran an RT PCR to test for exon 18 inclusion (Figure 9A). In SRSF1 and SRSF7 KO cells, there was a significant increase in exon 18 inclusion reaching 41.34% ± 11.87 and 37.17% ± 7.681, respectively, compared to the control of 16.39% ± 3.723 ($p= 0.0076$; $p= 0.0322$; Figure 9B). Exon 18 inclusion also decreased in SRSF3 KO cells, but the trend was not significant ($p= 0.1461$). Furthermore, hnRNPHI/H also showed a possible decrease in exon 18 inclusion, but more trials must be run to test for significance. Using a similar splicing factor panel, we were unable to significantly change exon 18 inclusion through SR protein over expression (Figure 10B). Even though OE studies showed no significant targets, SRSF1 and SRSF7 are still potential targets for genetic therapy an support our hypothesis that exon 18 splicing can be manipulated using known splicing factors.

A)



B)

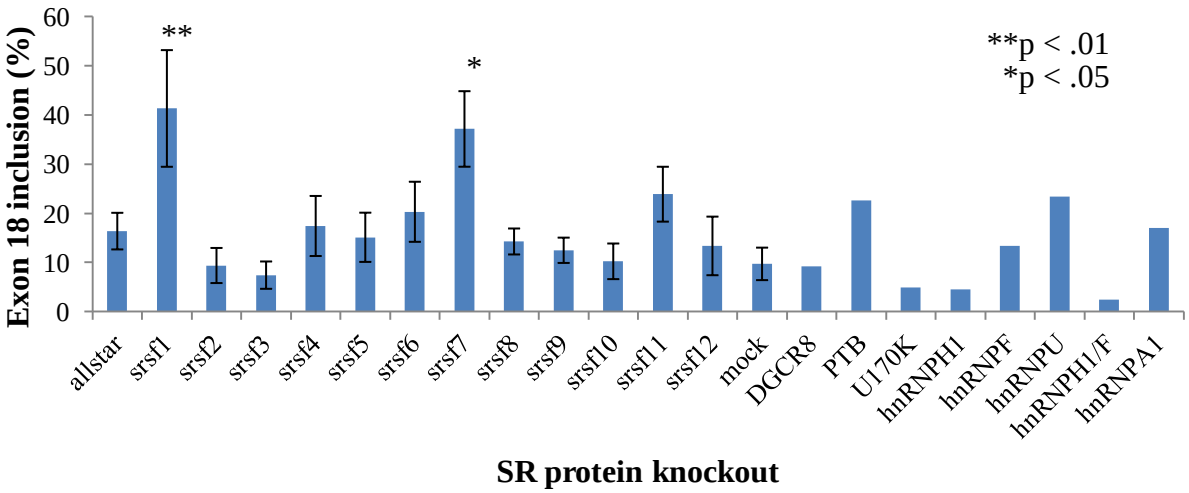
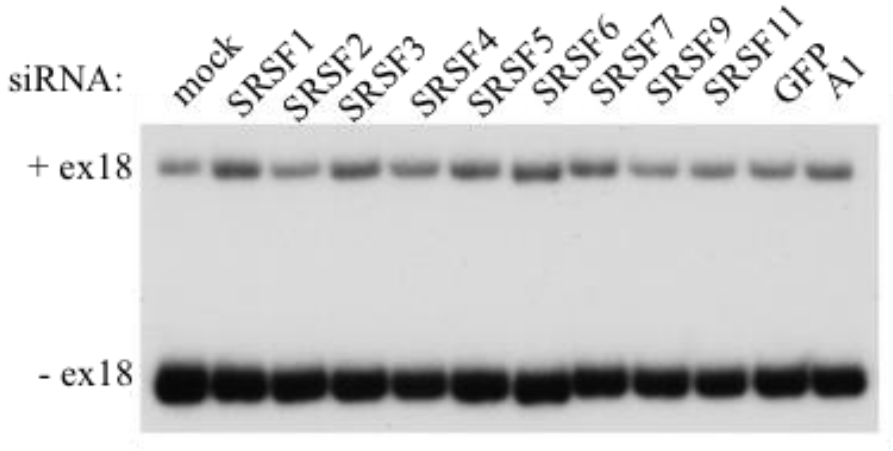


Figure 9. SR proteins regulate exon 18 splicing. (A) RT PCR of SR protein knockout 293T cells was performed; run on a 6% acrylamide gel with ^{32}P-dCTP. Upper bands include exon 18, and lower bands skip the alternatively spliced exon. **(B)** Average exon 18 % inclusion of three samples was calculated. SRSF1 and SRSF7 knockouts promoted exon 18 inclusion (**p< 0.01, *p< 0.05).

A)



B)

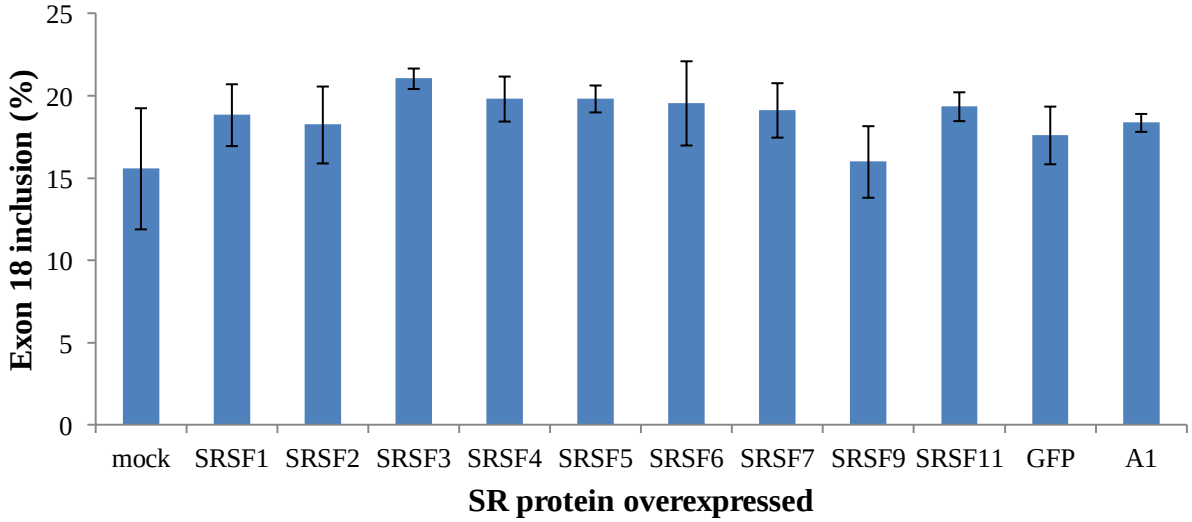


Figure 10. SR protein over expression does not significantly affect exon 18 inclusion. **(A)** RT PCR of siRNA overexpressed 293T cells were run on a 6% acrylamide gel with ^{32}P-dCTP. Upper bands include exon 18, and lower bands skip the alternatively spliced exon. **(B)** Average exon 18 inclusion of two samples showed no significant change with any overexpressed SR proteins.

Discussion

Given that AD prevalence continues to increase in today's world, studying this disease is of the utmost importance. Studying both the underlying mechanisms and causes of the disease as well as early biomarkers and treatments adds to science's potential to solve a growing problem. While current treatments do not prove effective for a large portion of AD patients, nearly 100 new treatments are presently being tested. AD treatment is most effective during the earliest stages of the disease, therefore early detection is important for any potential AD therapy. However, no AD biomarker capable of tracking AD progression exists. Aside from the potential benefits to AD treatment, studying precursory changes to identify new biomarkers also lends to current knowledge of AD disease mechanisms and may allow the creation of a genetic therapy for this devastating disease because no successful genetic therapy currently exists.

While the exact mechanisms underlying AD are still ambiguous, research has identified several risk factors involved, including ApoE. Studying ApoE isoforms in AD patients directed scientists towards an ApoE receptor, ApoER2, primarily involved in the reelin pathway. Current research implicates the reelin pathway as a possible mechanism being deregulated in AD and contributing to the progression of the disease. The purpose of my research was to study the relationship between the alternative splicing of ApoER2 exon 18 and AD progression and attempt to regulate splicing by targeting splicing factors. We hypothesized that exon 18 inclusion in neurons decreases as AD state progresses. Preliminary research supported our hypothesis and showed that ApoER2 exon 18 inclusion decreased as AD progressed. My research found that 1) ApoER2 exon

18 inclusion is decreasing in AD neurons, 2) hippocampal ApoER2 exon 19 inclusion decreases with age in AD mice, and 3) targeting SR protein splicing factors can increase exon 18 inclusion.

ApoER2 exon 18 inclusion decreases in human AD neurons

While our preliminary research returned promising results, it was possible that the decrease in exon 18 inclusion found in human mid-temporal AD brain samples was a result of a change in neuronal populations due to AD neuronal death (Coleman & Flood, 1987). The measured decrease in exon 18 inclusion could have been a result of measuring glial cell ApoER2 exon 18 inclusion, which may be different than that of neurons, in AD patients. To address this, we correlated exon 18 percent inclusion with glial cell populations using MBP relative RNA abundance as a marker for glial cells. There was no correlation between MBP relative RNA abundance and exon 18 inclusion in the human mid-temporal brain samples showing that the splicing change being measured did not occur due to a shift in neuronal and glial cell populations. This supports our hypothesis that a decrease in exon 18 inclusion occurs in AD patients' neurons.

The measured change in hippocampal exon 18 inclusion supports a change occurring in neurons before or independent of cell death. Additionally, this initial change may lead to neuronal death in AD. Understanding precursory changes, such as the decrease of exon 18 inclusion, gives insight into possible disease mechanisms and how potentially deregulated pathways may be involved. The ability to measure a precursory change in AD would allow for early detection through the use of AD biomarkers, and

early detection could then increase therapeutic success. Measuring this change also identifies a prospective therapeutic target for AD.

Hippocampal ApoER2 exon 19 inclusion decreases with age in AD mice

After finding support for a decrease in exon 18 inclusion in human AD neurons, we chose a second model in which to study the disease. TAS transgenic AD mice are one of several mouse models for AD. TAS mice develop plaques around 6 months of age and have drastically increased levels of $A\beta_{42}$ in the hippocampus and cortex (Pugh, et al., 2007; Holcomb, et al., 1998). As a result, TAS mice resemble AD most closely; thus, we chose to study this model both before and after plaque formation is expected in order to study AD progression.

Using the observed splicing change in human neurons, we hypothesized that exon 19 inclusion would decrease in this model as well. In support of our hypothesis, exon 19 inclusion decreased in the hippocampus of AD mice as they aged. There was a significant 22.07 % decrease in exon 19 inclusion in the hippocampus of 5-7 month AD mice compared to 1-2 month AD mice. AD pathology, specifically plaques and tangles, have been found to increase in the hippocampus as cognitive impairment and AD state increase in humans, therefore we expected for the hippocampus in TAS mice to be most affected by age (Sabuncu, 2011).

For a biomarker measuring splicing to be effective, it is integral that a splicing change be seen in peripheral body tissue or fluid such as blood or CSF (Biomarkers on a roll, 2010). For this reason, other cortical areas in TAS mice were tested in order to

address whether the change in exon 19 inclusion is area specific or universal. There was no significant change in exon 19 inclusion in the cerebellum or cortex of AD or control mice. Both AD patients and TAS mice tend to develop AD characteristic in the hippocampus earlier in the progression of the disease which would cause greater changes in exon 19 splicing were expected in the hippocampus rather than the cerebellum or cortex. Still, the lack of a universal change lends to the possibility that a change in ApoER2 alternative splicing may not serve as a useful AD biomarker. Regardless, no conclusion may be drawn until other bodily fluids are tested.

An increase in exon 19 inclusion was also observed in the hippocampus of control mice as they aged and a significant difference in exon 19 inclusion between control and AD mice at 1-2 months was observed. In control mice, the increase in exon 19 inclusion may indicate that the exon's importance may increase with age in healthy brains. However the significantly higher inclusion of exon 19 in young AD mice compared to control mice may occur as an attempt to overcompensate for AD deficiency or reelin pathway deregulation at young ages; a process that may fail to regulate the pathway believed to be deregulated as AD mice age, which is supported through reelin's presence in the Aβ plaques of TAS mice (Wirths, 2001). The overall increased level of exon 19 inclusion in young AD mice may also serve as an area of interest for future research because it would allow scientists to assess the effect of this level of inclusion on neurons.

Targeting SR protein splicing factors can increase exon 18 inclusion

For a change in exon 18 inclusion to occur, a change in alternative splicing via the

spliceosome must take place. A delicate balance must exist between ESEs, ESSs, ISEs, and ISSs, and by manipulating these various splicing regulators, exon 18 inclusion may be altered (Zhu, et al., 2001; Wagner & Garcia-Blanco, 2001). Thus, we hypothesized that exon 18 inclusion could be manipulated by targeting a panel of known splicing factors. Supporting our hypothesis, we were able to manipulate exon 18 inclusion in HEK 293T cells using by knocking out splicing factors.

When SRSF1 and SRSF7 were knocked-out, exon 18 inclusion significantly increased, but knocking-out the other SR proteins tested did not affect exon 18 inclusion significantly. When another two SR proteins, SRSF3 and hnRNPHI/H, were knocked-out, exon 18 inclusion appeared to decrease, but more trials must be completed to assess the ability of these splicing factors to affect inclusion significantly. When all of these splicing factors were overexpressed, exon 18 inclusion was not affected. However, while over expressing SRSF1 and SRSF7 did not decrease inclusion as we expected, they would still be ideal splicing factors to begin studying for potential gene therapies.

The main issue with targeting splicing factors is the sheer quantity of splicing factors in transcripts; targeting SR proteins can be successful, but it may also affect multiple transcripts and therefore have a pleiotropic effect. Each SR protein targeted recognizes a specific sequence on ApoER2's pre-mRNA. By targeting the SR protein binding sequences we are able to modulate their activity in a subtle way. This has been done using nucleic acid decoy strategies to target transcription factors, which bind to DNA in a manner similar to SR protein binding to RNA (Ishizaki et al., 1996). Makeyev et al. (2002) used small mRNA decoys to target various mRNA posttranscriptional controls. The were able to use a decoy cassette with snRNA's to deliver the RNA decoys

in vivo while maintaining high affinity binding to the RNA-binding protein.

Limitations and future studies

It would be beneficial to increase the sample size of both the human samples and AD mice tested in an attempt to further dissect the trends found in ApoER2 alternative splicing. Larger sample sizes would allow for more robust statistical tests. Furthermore, it would be particularly important to increase the number of control mice because they account for only a third of the total samples. An increased number of control mice would allow us to further study the unexpected trend discovered, and the increase in exon 19 inclusion in control mice could lead to an explanation of the necessity of higher ApoER2 activity or reelin signaling throughout the aging process. Understanding this mechanism could also lead to a possible AD therapy through the correction of the reelin pathway in AD patients.

In this study, only correlations could be made between the AD model and splicing. This study does not specify whether the change in ApoER2 splicing causes AD progression, or if the change is caused by the progression of the disease. To identify the direction of this relationship, exon 19 must be directly manipulated in the mouse model. RNA decoys, similar to those described by Makeyev et al. (2002), can be used to bind to and knock-out SR proteins that promote exon 19 inclusion in the mouse model. The mouse can then be observed for the development of an AD phenotype. RNA decoys can also be used in a similar manner to knock-out splicing factors in order to increase exon 19 inclusion in mice. If this rescues AD mice, then the treatment would need further

assessment as an AD therapy for humans.

Regardless of the direction of the correlation between AD progression and ApoER2 splicing, the change in ApoER2 splicing may still be a useful marker if it can be observed in peripheral tissue or fluid. If the change in splicing precedes the disease, then targeting exon 18 inclusion may delay AD onset, progression, or severity. Conversely, if the change in ApoER2 splicing follows the onset of AD, it can still be used as a potential measure of AD progression.

Scientists have been able to identify both Aβ and Tau levels in CSF as markers identifying a change in AD condition from MCI to AD, but this test is still not an efficient way in which doctors can test AD patients early enough to treat AD effectively (Williams, 2011). If no single marker for disease progression can be found in the blood or CSF of AD patients, it is possible to use a combination of markers. Ray et al. (2007) used this technique to come to the conclusion that a series of 18 proteins in the blood can be used to differentiate between disease states. However, a more efficient and accurate method may still exist. In combination with genetic tests for other AD genetic risk factors such as ApoE, a decrease in exon 18 inclusion may indicate the necessity to begin treatment in AD patients.

Generally AD patients do not have as severe a form of AD as the mouse models we used because we tested the most severe model available due to a double mutation of both APP and PS-1. Most AD patients do not suffer from the multiple mutations that characterize this aggressive form of AD. While it is beneficial to use an aggressive form in our model to allows us to measure alternative splicing both before and after plaque formation and disease onset, it may also be important to test a less severe model with

only one mutation. Studying a PS-1 mutant mouse model would also us to test for the mutation when $A\beta_{42}$ is slightly elevated, but no pathology is present which may be similar to an earlier stage of AD (Holcomb, et al., 1998). Additionally, an APP transgenic model, which only begins to develop AD pathology after about nine months, may allow for an increased observation of the progression of the disease in its earlier stages (Games, et al., 1995).

Observing AD mice in the earlier stages of the disease may also serve as a suitable model to test RNA decoy therapies. However, the method used in this study, targeting splicing factors using siRNAs, is not the best method when attempting to over express the SR proteins because the knocked-out and overexpressed SR proteins could be indirectly affecting the alternative splicing of exon 18. An in vitro splicing assay and mutation deletion analysis can also be performed in the future to assess this relationship between splicing factors such as SRSF1 or SRS7 and AD progression. Figure 11 shows the possible relationship between splicing factors, pathology at the synapse, and overall AD phenotype. In healthy neurons, it is possible that there are higher levels of SRSF3 or low levels of SRSF1/7, which could lead to increased exon 18 inclusion and allow reelin to antagonize Aβ at the synapse and prevent NMDAR endocytosis— which leads to a normal healthy phenotype (Figure 11A). Unlike healthy neurons, AD neurons may have low levels of SRSF3 or high levels of SRSF1/7, which could lead to decreased exon 18 inclusion. In turn, this may prevent reelin's ability to antagonize the already increased levels of Aβ at the synapse which may lead to increased NMDAR endocytosis and an overall AD phenotype (Figure 11B).

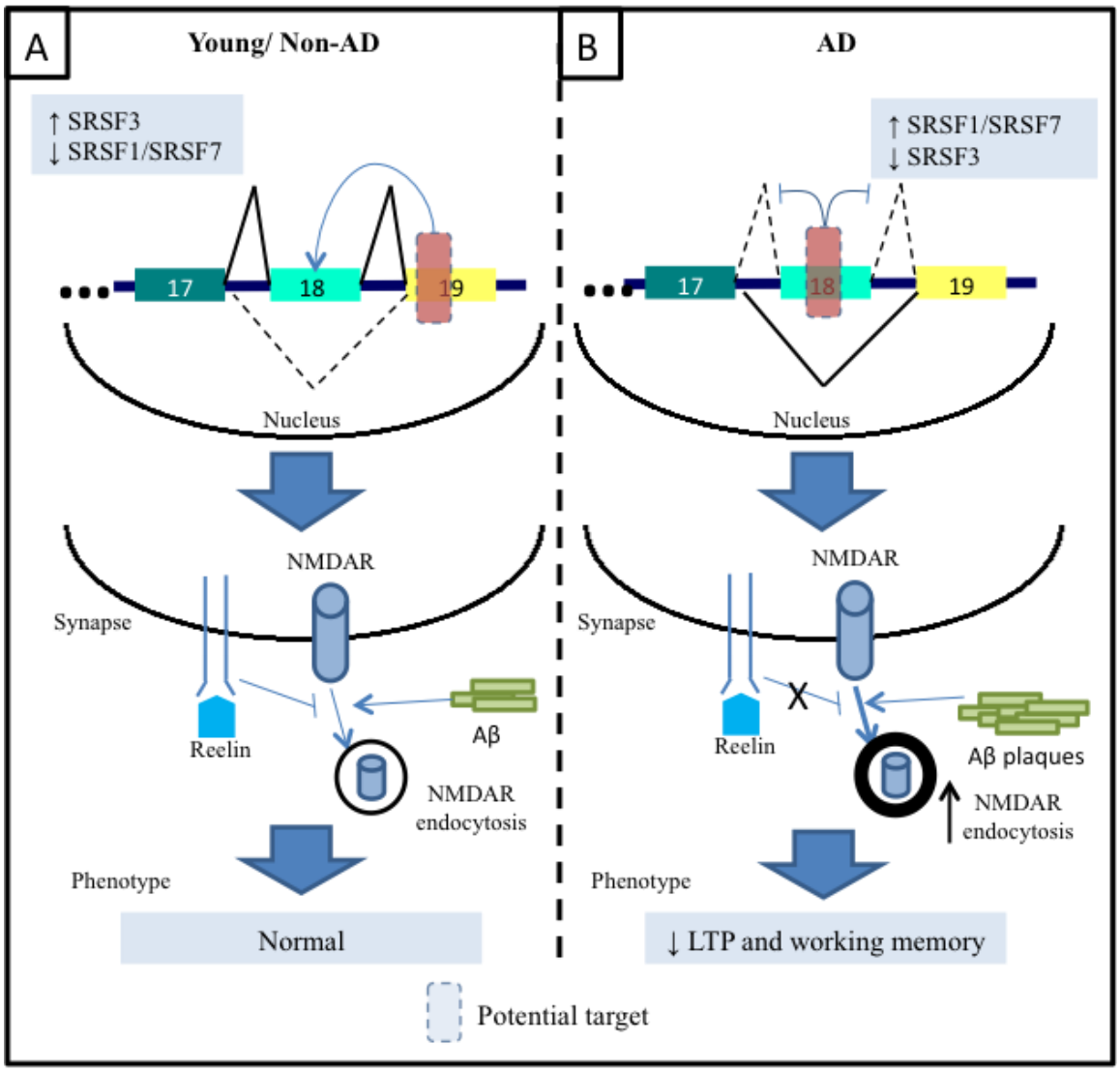


Figure 11. ApoER2 exon 18 inclusion affects overall AD phenotype and SR proteins may be used as therapeutic targets to enhance inhibition of SNDAR endocytosis. (A) Healthy cells may over express SRSF3 and/or under express SRSF1 or SRSF7 to promote healthy levels of exon 18 inclusion. This increases reelin's ability to antagonize Aβ at the synapse and prevent NMDAR endocytosis. This allows for proper synaptic function and a normal phenotype. **(B)** In AD neurons, SRSF3 may be under expressed and/or SRSF1 or SRSF7 may be overexpressed to decrease exon 18 inclusion. This may lead to reelin's inability to effectively antagonize Aβ at the synapse, thus promoting NMDAR endocytosis and synaptic dysfunction that leads to deficits in LTP and working memory. By targeting these splicing factors in potential genetic therapies in the future, we may be able to inhibit the incorrect splicing of exon 18 and thus restore AD neurons to a healthy phenotype.

It is also important to see if how Aβ processing is being affected by the change in exon 18 inclusion. Using the splicing factors that were most effective in manipulating exon 18 inclusion when they are knocked-out or overexpressed, such as SRSF1 or SRSF7, and an ELISA assay, extracellular Aβ could be measured. Ideally, knocked-out or overexpressed sequences that increase inclusion, should be able to decrease the amount of extracellular Aβ. These studies can also be performed if a tau related gene is found to be alternatively spliced in AD.

Conclusion

The purpose of this study was to test for an association between the alternative splicing of ApoER2 exon 18 and AD in neurons, which we hypothesized to be positively correlated. This association may serve to elucidate a possible underlying mechanism of dysfunction in AD, a biomarker, or potential targets for genetic therapy. We found that exon 18 inclusion decreased in AD patient neurons. Furthermore, this change was also seen in the hippocampus of a TAS AD mouse model with an APP/PS1 mutation. In TAS mice, this change was area selective rather than universal because splicing did not change in the cerebellum or cortex. This leads to the discovery that ApoER2's may not be an ideal AD biomarker. Yet, more studies must be done to test peripheral tissues and fluids for a change in ApoER2 exon 18 inclusion.

After detecting the AD associated decrease in exon 18 inclusion in humans and an animal model, an *in vitro* cellular model was used to target exon 18 inclusion. SR protein sequences were targeted using siRNAs, and several potential splicing factors of interest were identified. SRSF1 and SRSF7 may inhibit exon 18 inclusion while SRSF3 and hnRNPHI/H may promote its inclusion. These splicing factors are areas for future research for potential therapeutic targets. Overall, this research also lends an insight into a possible deregulated pathway in AD: the reelin pathway. While reelin has already been identified as an antagonist for Aβ at the synapse, more research must be performed to assess it greater role in AD and the significance of the pathway's decrease in exon 18 inclusion that is seen in AD.

Appendix A: Mouse demographics

Group	Strain	Animal	Sex	Age
1-2 mo	TAS	**2071**	M	7 wk
		2072	M	7 wk
		2073	F	7 wk
		2074	F	7 wk
		2094	M	7 wk
		2095	M	7 wk
		2096	F	7 wk
		401	M	6 wk
		402	M	6 wk
		403	F	6 wk
		404	F	6 wk
	NonTg	**2087**	M	7 wk
		2088	M	7 wk
		2089	F	7 wk
		2090	M	6 wk
		2091	M	6 wk

Group	Strain	Animal	Sex	Age
5-7 mo	TAS	**2060**	M	6 mo
		2061	F	6 mo
		2064	M	7 mo
		2065	M	7 mo
		2066	M	6 mo
		2067	M	6 mo
		405	M	5mo
		406	M	5 mo
		407	F	5 mo
	NonTg	**2063**	F	5 mo
		2079	M	22 wk
		2080	M	22 wk
		2081	F	22 wk
		2082	F	22 wk
		2092	M	25 wk
		2093	F	25 wk

Appendix B: Formulas

Amplification efficiency = (R2/R1)^(1/(average R2 CT value - average R1 CT value))-1

Relative RNA quantity = R1 CT value/ (amplification efficiency +1)^R1 CT average

Literature Cited

Alzheimer, A. (1907). Über eine eigenartige Erkrankung der Hirnrinde. *Allgemeine Z Psychiatrie Psychisch-Gerichtliche Medizin*, 146-148.

Azevedo, F., Carvalho, L., Grinberg, L., Farfel, J., Ferretti, R., Leite, R., Lent, R. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *The Journal of Comparative Neurology*, 532-541.

Beffert, U., Weeber, E., Durudas, A., Qiu, S., Masiulis, I., Sweatt, J., Herz, J. (2005). Modulation of synaptic plasticity and memory by Reelin involves differential splicing of the lipoprotein receptor ApoER2. *Neuron*, 567-579.

Biomarkers on a roll. (2010). *Nature Biotechnology*, 431.

Brandes, C., Kahr, L., Stockinger, W., Hiesberger, T., Schneider, W., & Nimpf, J. (2001). Alternative splicing in the ligand binding domain of mouse ApoE receptor-2 produces receptor variants binding reelin but not alpha 2-macroglobulin. *Journal Biological Chemistry*, 22160-22169.

CDC. (2011). *2011 Alzheimer's Disease Facts and Figures.* Special Report: Early Detection and Diagnosis.

Citron, M., Westaway, D., Weiming, X., Carlson, G., Diehl, T., Levesque, G., Selkoe, D. J. (1997). Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid -protein in both transfected cells and transgenic mice. *Nature Medicine*, 67-72.

Coleman, P. D., & Flood, D. G. (1987). Neuron numbers and dendritic extent in normal aging and Alzheimer's disease. *Neurobiology of Aging*, 521-545 .

Corder, E., Saunders, A., Strittmatter, W., Schmechel, D., Gaskell, P., Small, G., . . . Pericak-Vance, M. (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer’s disease in late onset families. *Science*, 261, 921-923.

Doody, R., Stevens, J., Beck, C., Dubinsky, R., & Kaye, J. (2001). Practice parameter: Management of dementia (an evidence-based review). Report of the quality standards subcommittee of the American academy of neurology. *Neurology*, 56 (9), 1154-1166.

Durakoglugil, M. S., Chen, Y., White, C. L., Kavalali, E. T., & Herz, J. (2009 йил 15-September). Reelin Signaling antagonizes B-amyloid at the synapse. *PNAS, 106*(37), 15938-15943.

Esch, F., Keim, P., Beattie, E., Blacher, R., Culwell, A., Oltersdorf, T.,Ward, P. (1990).

Science, 1122-1124.

Finder, V., & Glockshuber, R. (2007). Amyloid-beta aggregation. *Neurodegenerative. Dis.*, 13-27.

Fotuhi, M., Hachinski, V., & Whitehouse, P. J. (2009). Changing perspectives regarding late-life dementia. *Nature*, 649-458.

Galindo, M. F., Ikuta, I., Zhu, X., & Casadesus, G. (2010). Mitochondrial biology in Alzheimer's disease pathogenesis. *Journal of Neurochemistry*, 933-945.

Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Gillespie, F. (1995). Alzheimer-type neuropathology in transgenic mice over expressing V717F beta-amyloid precursor protein. *Nature*, 523-527.

Gong, Y., & Lippa, C. F. (2010). Disruption of the Postsynaptic Density in Alzheimer's Disease and Other Neurodegenerative Dementias. *American Journal of Alzheimer's Dis. Other Dementias*, 547-555.

Graveley, B. (2000). Sorting out the complexity of SR protein functions. *RNA*, 1197-1211.

Grear, K. E., Ling, I.-F., Simpson, J. F., Furman, J. L., Simmons, C. R., Peterson, S. L., Estus, S. (2009). Expression of SORL1 and a novel SORL1 splice variant in normal and Alzheimers disease brains. *Molecular Neurodegeneration, 4*, 46.

Guo, L., LaDu, M., & Van Eldik, L. (2004). A dual role for apoliprotein e in neuroinflammation: anti- and pro-inflammatory activity. *Journal of Molecular Neuroscience*, 205-212.

Halushka, M., Fan, J., Bentley, K., Hsie, L., Shen, N., Weder, A., Chakravarti, A. (1999). Patterns of single-nucleotide polymorphism in candidate genes for blood-pressure homeostasis. *Nature Genetics. 22*, 239-247.

Hebert, L., Scherr, P., Bienias, J., Bennet, D., & Evans, D. (2003). Alzheimer's disease in the U.S. population: prevalence estimates using the 2000 census. *Archives Neurology, 60*, 1119-1122.

Hering, H., & Sheng, M. (2001). Dendritic spines: structure, dynamics and regulation. *Nature Review Neuroscience, 2*(12), 880-888.

Herrup, K. (2010). Reimagining Alzheimer's disease-An age-based hypothesis. *Journal Neuroscience.*, 16755-16762.

Herz, J., & Chen, Y. (2006). Reelin, lipoprotein receptors and synaptic plasticity. *Nature*,

850-858.

Hibi, T., Mizutani, M., Baba, A., & Hattori, M. (2009). Splicing variations in the ligand-binding domain of ApoER2 results in functional differences in the binding properties to Reelin. *Neuroscience Res*, 251-258.

Hiesberger, T., Trommsdorff, M., Howell, B. W., Goffinet, A., Mumby, M. C., Cooper, J. A., & Herz, J. (1999). Direct binding of reelin to VLDLR receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates Tau Phospohrylation. *Neuron*, 481-489.

Hoe, H., Tran, T., Matsuoka, Y., Howell, B., & Rebeck, G. (2006). Dab1 and Reelin effects on APP and ApoER2 trafficking and processing. *Journal Biological Chemistry. 281*(46), 35176-35185.

Hoe, H.-S., Pocivavsek, A., Chakraborty, G., Fu, Z., & Vicini, S. (2006). Apoliprotein E Receptor 2 Interactions with the N-Methyl-D-asparate Receptor. *Journal of Biological Chemistry*, 3425-3431.

Holcomb, L., Gordon, M. N., McGowan, E., Yu, X., Benkovic, S., Jantzen, P., . . . Duff, K. (1998). Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nature Medicine, 4*(1), 97-100.

Howell, B., Herrick, T., & Cooper, J. (1999). Reelin-induced tyrosine phosphorylation of disabled 1 during neuronal positioning. *Genes Developmental.*, 643-648.

Howlett, D. R., Richardson, J. C., Austin, A., Parsons, A. A., Bate, S. T., Davies, D. C., & Gonzalez, M. I. (2004). Cognitive correlates of AB deposition in male and female mice bearing amyloid precursor protein and presenilin-1 mutant transgenes. *Brain Research*, 130-136.

Ishizaki, J., Nevins, J. R., & Sullenger, B. A. (1996). Inhibition of cell proliferation by an rna ligand that selectively blocks e2f function. *Nature Medicine*, *2*, 1386-1389.

Kar, S., Slowikowski, S., Westaway, D., & Mount, H. (2004). Interactions between B-amyloid and central cholinergic neurons: Implications for Alzheimer's disease. *J. Psychiatry Neuroscience*, 427-441.

Khairallah, M. I., & Kassem, A. A. (2011). Alzheimer's disease: Current status of etiopathogenesis and thereputic strategies. *Pakistan Journal of Biological Sciences*, 252-272.

Lahiri, D., Sambamurti, K., & Bennett, D. (2004). Apolipoprotein gene and its interaction with the environmentally driven risk factors: Molecular, genetic and epidemiological studies of Alzheimer's disease. *Neurobiol. Aging*, 651-660.

Loo, D., Copani, A., Pike, C., Whittemore, E., Walencewicz, A., & Cotman, C. (1993 йил 1-September). Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *PNAS, 90*, 7951-7955.

Ma, J., Yee, A., Brewer, H. J., Das, S., & Potter, H. (1994). Amyloid-associated proteins alpha I-antichymotrypsin and apoliprotein E promote assembly of Alzheimer beta-protein into filaments. *Nature*, 92-94.

Ma, S. L., Ng, H. K., Baum, L., Pang, J. C., Chiu, H. F., Woo, J., Lam, L. C. (2002). Low-density lipoprotein receptor-related protein 8 (apolipoprotein E receptor 2) gene polymorphisms in Alzheimer's disease. *Elsevier, 332*, 216-218.

Makeyev, A. V., Eastmond, D. L., & Liebhaber, S. A. (2002). Targeting a KH-domain protein with rna decoys. *RNA, 8*, 1160-1173.

Mayer, H., Duit, S., Hauser, C., Schneider, W., & Nimpf, J. (2006). Reconstitution of the Reelin signaling pathway in fibroblasts demonstrates that Dab1 phosphorylation is independent of receptor localization in lipid rafts. *Molecular Cell Biology*, 19-27.

Nakamura, Y., Yamamoto, M., & Kumamaru, E. (2001). Significance of the varient and full-length forms of the very low density lipoprotein receptor in brain. *Brain Research*, 209-215.

Niu, S., Yabut, O., & D'Arcangelo, G. (2008). The Reelin Signaling Pathway Promotes Dendritic Spine Development in Hippocampal Neurons. *The Journal of Neuroscience*, 10339-10348.

Pleckaityte, M. (2010). Alzheimer's disease: A molecular mechanism, new hypotheses and therapeutic strategies. *Med.*, 70-76.

Pugh, P. L., Richardson, J. C., Bate, S. T., Upton, N., & Stunter, D. (2007). Non-cognitive behaviours in an APP/PS1 transgenic model of Alzheimer's disease. *Behavioral Brain Research*, 18-28.

Ray, S., et al. (2001). Classification and prediction of clinical Alzheimer's diagnosis based on plasma proteins. *Nature Medicine*, 13: 1359- 1362.

Rebeck, G. W., LaDu, M. J., Estus, S., Bu, G., & Weeber, E. J. (2006). The generation and function of soluble apoE receptors in the CNS. *Molecular Neurodegeneration*, 15.

Rebeck, G., Reiter, J., Strickland, D., & Hyman, B. (1993). Apoliprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron*, 575-580.

Reitz, C., Honig, L., Vonsattel, J., & Mayeux, R. (2009). Memory Performance is Related to Amyloid and Tau Pathology in the Hippocampus. *J Neurol. Neurosurg. Psychiatry*, 715-721.

Rocci, A., Pellegrini, S., Siciliano, G., & Murri, L. (2003). Causative and susceptibility genes for Alzheimer's disease: A review. *Brain Research Bulletin*, 1-24.

Ruiz, J., Kouiavskaia, D., Migliorini, M., Robinson, S., Saenko, E., Gorlatova, N., Strickland, D. (2005). The apoE isoform binding properties of the VLDL receptor reveal marked differences from LRP and the LDL receptor. *Journal Lipid Research*, 1721-1731.

Sabuncu MR, D. R. (2011). The dynamics of cortical and hippocampal atrophy in Alzheimer disease. *Arch. Neurol.*, 1040-1048.

Selkoe, D. (1997). Alzheimer's disease: genotypes, phenotypes, and treatments. *Science*, 630-631.

Sinagra, M., Verrier, D., Frankova, D., Korwek, K. M., Blahos, J., Weeber, E. J., Chavis, P. (2005). Reelin, very-low-density lipoprotein receptor, and apolipoprotein E receptor 2 control somatic NMDA receptor composition during hippocampal maturation in vitro. *Journal of Neuroscience*, 6127-6136.

Stockinger, W. E. (2000). The reelin receptor ApoER2 recruits JNK-interacting proteins-1 and -2. *Journal of Biological Chemistry, 275*, 25625-25632.

Strittmatter, W., Weisgraber, K., Huang, D., Dong, L., Salvesen, G., Pericak-Vance, M., Roses, A. (1993). Binding of human apoliprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease. *PNAS*, 8098-8012.

Tissir, F., & Goffinet, A. (2003). Reelin and brain development. *Nature Review Neuroscience, 4*, 496-505.

Trommer, B., Shah, C., Yun, S., Gamkrelidze, G., Pasternak, E., Stine, W., . . . LaDu, M. (Dis). ApoE isoform-specific effects on LTP: blockade by oligomeric amyloid-beta I-42. *Neurobiol.*, 2005.

Wagner, E., & Garcia-Blanco, M. (2001). Polypyrimidine tract binding protein antagonizes exon definition. *Mol. Cell Biol.*, *21*(10), 3281-3288.

Walsh, D., & Selkoe, D. (2007). AB oligomers-a decade of discovery. *J. Neurochem.*, 1172-1184.

Weeber, E., Beffert, U., Jones, C., Christian, J., Forster, E., Sweatt, J., & Herz, J. (2002). Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity

and learning. *J. Biol. Chem.*, 39944-39952.

Williams, R. (2011). Biomarkers: Warning signs. *Nature*, 475: S5-S7.

Wirths O, M. G. (2001). Reelin in plaques of beta-amyloid precursor protein and presenilin-1 double-transgenic mice. *Neuroscience Letter, 316*(3), 145-148.

Yarri, R., & Corey-Bloom, J. (2007). Alzheimer's disease: Pathology and pathophysiology. *Semin. Neurol.*, 32-41.

Yasui, N., Nogi, T., Kitao, T., Nakano, Y., Hattori, M., & Takagi, J. (2007). Structure of a receptor-binding fragment of Reelin and mutational analysis reveal a recognition mechanism similar to endocytic receptors. *PNAS, 104*, 9988-9993.

Zhu, J., Mayeda, A., & Krainer, A. (2001). Exon identity established through differential antagonism between exonic splicing splicer-bound hnRNP A1 and enhancer-bound SR proteins. *Mol. Cell*, 1351-1361.

Zlokovic, B. (2004). Clearing amyloid through the blood-brain barrier. *J Neurochem*, 807-811.