Apolipoprotein E4 and SirT1 Interaction in Alzheimer’s Disease

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Apolipoprotein E4 and SirT1 interaction in Alzheimer’s Disease

A thesis submitted to the faculty of
Dominican University of California
& The Buck Institute of Aging
in partial fulfillment of the requirements
for the degree

Master of Science

In

Biology

By

Brittany Philpot

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2014
This thesis, written under the direction of the candidate’s thesis advisor and approved by the Chair of the Master’s program, has been presented to and accepted by the Faculty of Natural Science and Mathematics in partial fulfillment of the requirements for the degree of Master of Biology. The content and research methodologies presented in this work represent the work of the candidate alone.

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Abstract:

Alzheimer’s disease (AD) is a neurodegenerative disorder whose exact cause(s) are still unknown. Epsilon 4, an allele of apolipoprotein E (ApoE4), is currently the most important risk factor for Alzheimer’s disease. Individuals that inherit two copies of the ApoE ε4 allele have an approximately ten to thirty times increased risk of developing AD in comparison to persons not carrying this allele. This association may relate to ApoE4’s susceptibility to proteolysis and neurotoxicity. Amyloid precursor protein (APP) is involved with neurite extension and neurite retraction, and has been shown to function as a molecular switch with two separate cleavage patterns. Cleavage at the β-, γ-, and caspase sites results in the production of four neurotoxic, pro-apoptotic, pro-AD peptides: sAPPβ, Aβ, Jcasp, and C31. In contrast, anti-AD cleavage at the α-site produces two neuroprotective peptides: sAPPα and αCTF. Recent data have shown significantly lower sAPPα levels in cerebrospinal fluid of AD patients who possess one or more ε4 allele. Recent research studies have focused attention on Sirtuins. SirT1 plays a role in synaptic plasticity, learning and memory. Additionally, it has been demonstrated to reduce Aβ accumulation and elevate α-secretase activity. ApoE4 has been suggested to decrease SirT1. SirT1 suppresses AD in cells, primary neurons, and mouse models, by activating transcription of ADAM10, thus increasing the levels of the neuroprotective sAPPα, which is the pro-ligand peptide. In addition, overexpression of SirT1 causally promotes α-secretase activity and attenuates Aβ peptides generation in primary neuron cultures. Using various methods, SirT1 demonstrates having an effect on ApoE4 and may have an interaction either directly or indirectly.
Table of Contents:

Overview................................................................. 7-9

Specific Aims......................................................... 9-10

Background......................................................... 10-13

Results.............................................................. 13-22

  Cellular Localization of ApoE4............................ 13-15

  ApoE’s interaction with Sirtuins............................ 15-20

  Drugs that affect ApoE-SirT interaction............... 20-22

Discussion...................................................... 21-24

Research Design and Methods.............................. 24-26

References...................................................... 27-28

Table............................................................... 29
**Abbreviations:**

Alzheimer’s Disease: AD

Beta Amyloid: Aβ

Sirtuin1: SirT1

Apolipoprotein E4: ApoE4

Amyloid Precursor Protein: APP

Immunocytochemistry: ICC

Co-immunoprecipitation: Co-Ip
Overview:

Two main hallmarks of Alzheimer’s disease (AD) are the deposition of fibrillar amyloid β peptides (Aβ) in senile plaques of the brain and hyperphosphorylation of tau proteins, which lead to loss of synapses and dendritic spines (Wang et al., 2013 and Hashimoto et al., 2012). The amyloid precursor protein (APP) has been shown to function as a molecular switch: cleavage at the β, γ, and caspase sites results in the production of four pro-AD-peptides—sAPPβ (from which N-APP is derived), Aβ, Jcasp, and C31—that mediate neurite retraction, synaptic reorganization, and ultimately programmed cell death (Bredesen et al., 2010; Bredesen et al., 2006; Butterfield et al., 2010). In contrast, cleavage at the α site produces the trophic peptide sAPPα and the inhibitor of APP γ-site cleavage, αCTF (Bredesen, 2009). The decision between these two proteolytic pathways is governed at least in part by ligand binding: interaction with the axon guidance and trophic factor netrin-1 increases α-site cleavage, whereas interaction with the anti-trophin Aβ inhibits α-site cleavage and increases net production of the four neurite-retractive peptides (Bredesen, 2009) [Fig 1].

The apolipoprotein E4 (ApoE4) (chromosomal locus 19q13) is the single most important genetic risk factor associated with AD. This allele confers increased risk for sporadic as well as familial AD (Mahley et al., 2006; Roses, 1996). Individuals with two copies of the ApoE ε4 allele have an approximately eightfold increased risk of AD and have a significantly lower age of onset compared to AD patients not carrying this allele (Mahley et al., 2006; Roses, 1996). While the exact mechanism of ApoE4 toxicity remains unclear, recent data indicate that the greater risk of AD associated with the ApoE4 isoform might relate to ApoE’s susceptibility to proteolysis and neurotoxicity (Mahley et al., 2006). According to a recent study, the level of CSF sAPPα is...
significantly lower in AD patients possessing one or two ApoE4 alleles than in those not possessing the ApoE4 allele (Olsson et al., 2003). Therefore, it was of interest to determine

**Figure 1: APP acts as a molecular switch:** Alternative cleavage of APP at the β, γ and caspase sites leading to four peptides namely; Aβ, sAPPβ, Jcasp and C31 that mediate synaptic loss, neurite retraction, and ultimately programmed cell death; or cleavage at the α-site leading to two peptides namely sAPPα and αCTF that mediate neurite extension, synaptic maintenance and inhibit programmed cell death. Among the factors that mediate the decision between these two pathways are included trophic effects such as netrin-1 and anti-trophic effects such as Aβ peptide (Bredesen, 2009).

Recent studies point to SirT1, which belongs to the Sirtuin family of NAD-dependent deacetylases as being neuroprotective and SirT2 as being neurotoxic. SirT1 suppresses AD in a mouse model for this disease by directly activating transcription of ADAM10 and increasing the levels of the neuroprotective sAPPα (Donmez et al., 2010; Julien et al., 2009). Since one of the
mechanisms by which ApoE4 could trigger reduction in sAPPα levels is by inhibiting the proteolysis of APP at the α-site, it was of interest to determine the effect of ApoE isoforms on SirT1 and SirT2 expression. By identifying if this relationship is significant by various methods, we should gain insight into the underlying processes driving Alzheimer’s disease: Furthermore, this may help us to develop therapeutic remedies that can increase SirT1 levels, therefore increasing sAPPα and decreasing Aβ.

Specific Aims:

In order to further understand the mechanisms underlying Alzheimer’s disease, the interaction between ApoE and APP must be understood more clearly, and also we must understand the proposed relationship between ApoE and Sirtuins. If there is an interaction between ApoE4 and Sirtuins, then this discovery may give us the necessary data to discover whether SirT1 and SirT2 are important in AD, and, if so, how ApoE is affecting this proposed relationship. Three specific aims I intend to accomplish in order to answer the question about the relationship between ApoE4 and Sirtuins are:

AIM 1: Cellular localization of ApoE.

APP and ApoE have been shown to interact with each other in the cell, and identifying in which cellular compartment(s) this occurs should give us insight into how they interact.

AIM 2: Does ApoE interact with Sirtuins?

One of the mechanisms by which ApoE affects pro-AD vs anti-AD peptide formation is by affecting SirT1 or SirT2 levels. To understand this better, we will be looking at ApoE-SirT interaction by immunoprecipitations and immunocytochemistry.
AIM 3: Drugs that will affect ApoE-SirT interaction and increase sAPPα.

Our data thus far indicate that APP, ApoE and SirT1/2 all may be part of a network that is affected in AD. Thus our data provide a medium-throughput model for therapeutic candidate screening directed at this network of molecules that needs to be considered as a critical target for AD drug discovery.

**Background:**

Alzheimer’s disease (AD) is a progressive neurodegenerative disease of the central nervous system (Capsoni et al., 2002). AD affects about 5 million Americans, and by 2050, this is expected to increase to 13 million people. The cost of AD in the United States is approximately $200 billion annually. Among people who are 65 and older, 15% (1 in 7 people) are diagnosed with AD and for those who are above age 85, 50% (1 in 2 people) are diagnosed (Bonda et al., 2011). Neurofibrillary tangles and senile plaques are the two main hallmarks of the disease. Although not all aspects of AD are fully understood, research in the field has come across some risk factors and mediators but understanding of the pathogenesis remains incomplete, and there is no truly effective treatment. The senile plaques are predominantly composed of β-amyloid (Aβ). After the amyloid precursor protein (APP) is cleaved by β- and γ-secretase, Aβ is formed. One of the causes of early onset familial AD (FAD; Capsoni…) is mutations in APP that lead to extracellular deposition of Aβ, this is one cause of early onset familial AD (Capsoni et al., 2002). APP has been shown to function in synaptic transmission and plasticity as well as in cell adhesion and motility. When APP interacts with netrin-1, neurite extension occurs, but when it interacts with Aβ, neurite retraction takes place, and ultimately cell death (Lourenco et al., 2009). APP is produced in large quantities in neurons and is metabolized...
very rapidly. After sorting in the endoplasmic reticulum and Golgi, APP is delivered to the axon, where it is transported by fast axonal transport to synaptic terminals. On the cell surface, APP can be proteolyzed directly by \( \alpha \)-secretase and then \( \gamma \)-secretase, a process that does not generate A\( \beta \), or reinternalized in clathrin-coated pits into another endosomal compartment containing the proteases BACE1 and \( \gamma \)-secretase. The latter results in the production of A\( \beta \), which is then dumped into the extracellular space following vesicle recycling or degraded in lysosomes. This part is very rapid, as little APP is on the surface at any point in time. BACE1 is the neuronal \( \beta \)-secretase and following BACE1 cleavage and release of the sAPP\( \beta \) ectodomain, the APP C-terminal fragment is cleaved by the \( \gamma \)-secretase complex at one of several sites varying from +40 to +44 to generate A\( \beta \) and the APP intracellular domain (O’Brien et al., 2011).

**Figure 2: Neurofibrillary tangles and senile plaques in AD.** The neurofibrillary tangles are made up of hyperphosphorylated tau, which leads to synapse lose. The dominant material in the senile plaques is A\( \beta \).
Pathology of autosomal dominant AD is very similar to sporadic AD, including neurofibrillary tangles and microglial infiltration. There are 32 APP, 179 PSEN1 (presenilin 1 gene locus), and 14 PSEN2 gene mutations that result in early-onset, autosomal dominant, and fully penetrant AD. In APP, mutations cluster around the $\gamma$-secretase cleavage site, although the most famous APP mutation (APP-SWE) causes a change in amino acids adjacent to the BACE1 cleavage site. PSEN gene mutations (which give rise to proteins called presenilins, PS1 and PS2) predominantly alter the amino acids in their nine trans-membrane domains. The common thread to all these mutations is that they increase production of the less soluble and more toxic A\textsubscript{42} relative to A\textsubscript{40} (O’Brien et al., 2011).

Neurofibrillary tangles, the other hallmark of AD, are made up of hyperphosphorylated tau in the brain [Fig 2]. The phosphorylation of tau leads to synapse loss. A\beta has been linked to tau entering into dendrites, which is associated with the loss of synapses, spines, and microtubules (Wang et al.,2013 ). Apolipoprotein E (ApoE) is known to bind to A\beta in plaques of the brain. The ApoE gene has three isoforms, $\varepsilon$2, $\varepsilon$3, and $\varepsilon$4. Inheritance of two copies of the ApoE $\varepsilon$4 allele increases the risk for developing AD by 10-fold compared to the ApoE $\varepsilon$3/$\varepsilon$3 genotype (Hashimoto et al.,2012). ApoE2 has been shown to decrease the risk compared to the other alleles. It remains unclear how ApoE affects plaque morphology, but in AD patients it has been shown that ApoE4 increases plaque deposition compared to ApoE2 and ApoE3 in humans (Youmans et al.,2012).

Recent research studies have focused attention on Sirtuins and their role in AD. Seven sirtuin genes are encoded by the human genome, SirT1 is the most well known. SirT1 which was first identified in yeast is a NAD(positive)-dependent enzyme that is a stress-response protein and helps mammals adapt to dietary manipulations. Sirtuins function by removing acetyl
groups from lysines while hydrolyzing NAD. SirT1 is predominantly found in the nucleus but has been reported to shuttle between the nucleus and the cytoplasm. SirT2 has been identified in the cytoplasm. SirT1 plays a role in synaptic plasticity, learning, and memory (Bonda et al., 2011). Additionally, it has been demonstrated to reduce Aβ accumulation and elevate α-secretase activity. In another study, SirT1 was shown to protect against microglia-dependent Aβ toxicity in cells through inflammatory NF-kB signaling (Lalla and Donmez, 2013). In contrast, SirT2 binds and deacetylates the transcription factor, FOXO3a, which leads to cell death under oxidative stress (Albani et al., 2010). These two types of sirtuins may have a purpose in Alzheimer’s disease based on the levels found in the brain of each one. Recently, Dr. Rammohan Rao, and colleagues discovered that ApoE4 expression decreases SirT1 and increases SirT2 levels (Theendakara et al., 2013), which is associated with oxidative stress, inflammation, and diabetes mellitus. These in turn are all risk factors in Alzheimer’s disease (Albani et al., 2010).

**Results:**

1. **Cellular localization of ApoE4:** A172 cells were either left untransfected (Figure 3A) or transfected with ApoE4 using lipofectamine (Figure 3B). Both sets of cells were fixed to an 8-well chamber slide and permeabilized in 0.3 % Triton. Both were stained with Alexa fluor 488 (green) and with DAPI (red/pink). The Alexa fluor 488 was administered at a 1:300 dilution. Using a confocal microscope, the images fluorescence were viewed and imaged. As shown in Fig 3, untransfected cells displayed some diffuse distribution of ApoE4 in the cytoplasmic region, however after transfection, ApoE4 staining was more intense and this increased staining was associated with the nucleus as well (Fig 3 B & C).
Figure 3: Localization of ApoE4 by immunocytochemistry. ApoE4 was found to be in the nucleus and cytoplasm in A172 cells before (A) and after transfection with ApoE4 (B). For the detection of ApoE, cells were stained with a mouse monoclonal anti-ApoE antibody as primary antibody and 488-conjugated donkey antimouse IgG as the secondary antibody (green). Nuclei (pink/red) are counterstained using VectaShield Mounting Medium with DAPI. Staining was observed at 63X objective with confocal microscope. (C). Using the Zen 2011 software, co-localization was viewed by showing yellow where ApoE4 is seen in the nucleus.
Cellular localization of ApoE4 was also determined by subcellular fractionation studies. ApoE3 and ApoE4 were transfected into A172 cells and subcellular fractions were obtained using a combination of specific buffers. Fractionation of the nucleus and cytoplasm was performed on these cells followed by SDS/PAGE and western blotting. PARP was used as a control for the nucleus to ensure that the extracts were pure. In addition to being predominantly in the cytoplasm both ApoE3 and E4 were also seen to be present in the nucleus [Fig 6A].

The exact pathway by which ApoE, which is normally destined for secretion, is able to reach and enter the nucleus remains unknown. Our results showing ApoE presence in the nucleus suggested that an active transport pathway may be involved, rather than a diffusion-based mechanism since large proteins (>30 kDa) require an active transport pathway largely mediated by importins (Yoneda, Y., (2000). Nucleocytoplasmic trafficking also requires the presence of NLSs (nuclear localization sequences) that are short stretches of basic amino acids. ApoE does contain two polybasic domains that fulfill the criterion for a potential ‘weak’ NLS (Saito, H., et al, 2003).

2. ApoE’s interaction with Sirtuins: Recent studies have shown that SirT1 which belongs to the Sirtuin family of NAD-dependent protein deacetylases, suppresses AD-related biochemical events in cells, primary neurons, and mouse models by directly activating transcription of ADAM10, thus increasing the levels of the neuroprotective sAPPα (Donmez G, et al, 2010). We decided to investigate the role of ApoE on SirT expression. As shown in Fig 4a, ApoE4 but not ApoE3, significantly down-regulated SirT1 gene expression without having any significant effect on SirT2 or SirT6 expression. Furthermore, the presence of either ApoE3 or ApoE4 resulted in a significant
reduction in SirT1 protein levels (Fig. 4b).

In contrast, while the presence of ApoE3 resulted in a commensurate reduction in SirT2 levels, the presence of ApoE4 triggered a ~2-fold increase in SirT2 expression. Thus the ratio of SirT2:SirT1 was unaffected by ApoE3, but increased markedly—approximately 4.5-fold—by the
Figure 5: **Effect of ApoE on cellular localization of Sirtuin.** A172 cells were either left untransfected (A) or transfected with SirT1 in the absence of ApoE4 (B) or together with ApoE4 (C) using lipofectamine. Cells were fixed to an 8-well chamber slide and permeabilized in 0.3 % Triton. Cells were stained with Alexa fluor 488 (green) and with DAPI (red/blue). The Alexa fluor 488 was administered at a 1:300 dilution. Using a confocal microscope, the images fluorescence were viewed and imaged.

expression of ApoE4 (Fig. 4b). Similar to the cell culture results, the SirT2:Sirt1 ratio was also increased in human AD subjects, although in these advanced cases, this effect was solely due to a reduction in SirT1 expression (Fig. 4c). Overexpression of SirT1 reversed the ApoE4-mediated reduction in sAPPα secretion and restored it to normal levels (Fig. 4d). Additionally,
overexpression of SirT1 reversed ApoE4-mediated decrease in sAPPα protein levels (Fig. 4e). To better understand the mechanism by which ApoE4 triggered reduction in SirT1 levels we looked at ApoE-SirT interaction and localization by immunoprecipitation and immunocytochemistry.

3. Effect of ApoE on cellular localization of Sirtuin: A172 cells were either left untransfected (Figure 5A) or transfected with SirT1 in the absence of ApoE4 (Figure 5B) or together with ApoE4 (Figure 5C) using lipofectamine. As shown in Fig 5A, untransfected cells displayed some nuclear and diffuse cytosolic distribution of SirT1. Increased staining in both cellular compartments was observed after transfection of SirT1 (Fig 5B). However after transfection of ApoE4, SirT1 staining was diminished considerably in both compartments suggesting a ApoE4-mediated reduction in SirT1 expression (Figure 5C).

We also confirmed the above-mentioned results with subcellular fractionation studies. SirT1 and ApoE3 or ApoE4 were transfected into A172 cells and subcellular fractions were obtained using a combination of specific buffers. Fractionation of the nucleus and cytoplasm was performed on these cells followed by SDS/PAGE and western blotting. As shown in Figure 6, SirT1 was seen only in the nucleus when ApoE3 is transfected into the cells, but in presence of ApoE4, SirT1 was shown to be both in the cytoplasm and the nucleus. The exact mechanism is still unclear, but the fractionation does display a clear difference between ApoE3 and ApoE4 effects on SirT1 (Figure 6A). This distinct difference may result from an interaction, direct or indirect, between both ApoE and SirT1.
Figure 6: **Cellular localization of Sirtuin by subcellular fractionation.** SirT1 and ApoE3 or ApoE4 were transfected into A172 cells and subcellular fractions were obtained using a combination of specific buffers. Fractionation of the nucleus and cytoplasm was performed on these cells followed by SDS/PAGE and western blotting. PARP was used as a control for the nucleus to ensure that the extracts were pure. SirT1 is present in both the nucleus and the cytoplasm when ApoE4 is present and is only in the nucleus when ApoE3 is present. PARP is used as a control for the nucleus. (B) **Co-immunoprecipitation of ApoE and SirT1.** Coimmunoprecipitation was carried out in A172 cells transfected with 6 µg of FLAG-SirT1 cDNA and ApoE3 or ApoE4 expression construct. 24h after transfection, cells were gently lifted and washed once with PBS at room temperature. Immunoprecipitations were performed with anti-Flag monoclonal antibody (to pull down Flag-tagged SirT1), and the resulting immunoprecipitates were subjected to SDS-PAGE and Western blotting using antisera specific for ApoE. Membranes were probed with anti-GAPDH antibody that served as a loading control. Lane 1 represents the extract loaded before the pull down to identify SirT1 and ApoE bands.

To determine if there was any interaction between ApoE and SirT1 at the protein level, FLAG-SirT1 cDNA was expressed following transfection of A172 cells. Immunoprecipitation was performed with anti-Flag monoclonal antibody (to pull down Flag-tagged SirT1) and the resulting immunoprecipitates were analyzed by immunoblotting using antisera specific for ApoE. As shown in Fig 6B, there was no evidence of direct interaction between SirT1 and
ApoE4 at the protein level, which leads us to believe that either there is an indirect interaction or ApoE-SirT interact at a protein-DNA level.

4. Drugs that will affect ApoE-SirT interaction and increase sAPPα. Given the effect of ApoE4 to reduce sAPPα levels and the reversal of this effect by SirT1, it would be of interest to identify drug candidates that return the SirT1 levels to normal in the presence of ApoE4.

A

B

Figure 7: Drugs that reverse ApoE4 effects on SirT1. A172 cells were transfected with ApoE4. Drug treatment was done at 2.5µM for 24 hours. (A) Real-time PCR was used to obtain SirT1 levels. The real-time PCR cycling was performed as described (Theendakara et al, Proc Natl Acad Sci; 110(45); 18303-8; 2013). Data (dCt values expressed as percentage of untransfected control) are from more than three experiments performed in triplicate (B) Twenty four hours after transfecting A172 cells with ApoE4, SirT1 enzyme activity was determined with the AlphaLISA immunoassay research kit (Perkin Elmer) according to the manufacturer’s protocol (Theendakara et al, Proc Natl Acad Sci; 110(45); 18303-8; 2013). Data (mean ± SE) are from four experiments performed in triplicate.
Such candidates would potentially prevent similar effects of ApoE4 in vivo. Therefore, candidates identified from an earlier library screen that successfully increased sAPPα levels were evaluated for their effects on SirT1 in the presence of ApoE4. A172 cells were transfected with ApoE4 in a 9-well plate. After 24 hours the drug candidates were administered at a 2.5µM concentration for 24 hours, and then the cells were harvested for assay. Real-time PCR was done to measure the content of SirT1 in each sample. All of these drug candidates showed an increase of SirT1 mRNA (Figure 7A). These drug candidates also showed an increase in SirT1 protein activity levels (Figure 7B).

Furthermore, we also screened our small molecule library for drugs that will reverse the ApoE4-mediated reduction in ADAM10 mRNA and sAPPα levels. A172 cells were transfected with ApoE4 in a 9-well plate. The cells were treated with different drugs at 2.5µM concentration for 24 hours. Using a real-time PCR the levels of ADAM-10 (which function as an α-secretase and thus produces sAPPα) were determined. The level of ADAM10 mRNA, which was decreased in the presence of ApoE4, was reversed by a subset of the drugs that were chosen (Figure 8A).

Similarly, there was a modest increase in sAPPα levels in the cells treated with the drugs compared to the cells that were transfected with ApoE4 (Figure 8B).

**Discussion:** Based on the work done so far by others in the lab and by me, our data reveal differential effects of ApoE4 vs. ApoE3 on APP interaction, signaling, and processing, and are compatible with the notion that the Alzheimer’s phenotype represents an imbalance between the trophic and anti-trophic signaling of APP, reflected by the ratio of the four APP-derived neurite-retractive peptides to the two APP-derived trophic peptides.
Figure 8: Drugs that reverse ApoE4 effects on sAPPα. A172 cells were transfected with ApoE4. Drug treatment was done at 2.5µM for 24 hours. (A) Real-time PCR was used to obtain ADAM10 levels. The real-time PCR cycling was performed as described (Theendakara et al, Proc Natl Acad Sci; 110(45); 18303-8; 2013). Data (ADAM10-δCt values expressed as percentage of untransfected control) are from more than three experiments performed in triplicate. (B) A172 cells were transfected with ApoE4. Twenty four hours after transfection, sAPPα secreted into the medium was determined with the AlphaLISA immunoassay research kit (Perkin Elmer) according to the manufacturer’s protocol with some modifications (Theendakara et al, Proc Natl Acad Sci; 110(45); 18303-8; 2013).
Our results indicate that ApoE4 interacts with high affinity with APP, shifting the processing balance in the anti-trophic direction, decreasing sAPPα secretion, and reducing sAPPα/Aβ and sAPPα/sAPPβ ratios in comparison to ApoE3.

In addition to these effects on APP processing and signaling, ApoE4 expression was also associated with a marked reduction in the ratio of SirT1 to SirT2, both in cultured neural cells and in the brains of patients with AD.

Our data may thus explain why the ApoE4 allele is the major risk factor or susceptibility gene associated with AD, and therefore represents an excellent target for AD drug discovery.

Our studies link for the first time the major risk factor for Alzheimer’s disease-ApoE4 with major longevity determinants, the Sirtuins; and identify the first candidate therapeutics that target this new link.

Our results are compatible with the notion that ApoE4-mediated signaling affects an endogenous program that mediates synaptic plasticity balance and may explain why ApoE4 allele is the major susceptibility gene associated with AD and should therefore be considered a critical target for AD drug discovery.

Thus far, our data indicate that ApoE4, p-APP, p-Tau, and SirT1 all may be part of a signaling network that is affected in AD, providing a medium-throughput model for therapeutic candidate screening in AD drug discovery.
**Research Design and Methods:**

*Immunocytochemistry (ICC):*

ICC is beneficial to answer the question of where ApoE localizes within the cell because this technique uses antibodies that target specific protein antigens. For cells, A172 cells, which are human glioblastoma cells, were used. These cells were fixed and then probed with an antibody in order to look at different cellular compartments that can be examined using a confocal microscope. These results allowed visualization of the cell where ApoE localizes and also if it reacts with APP in the nucleus or the cytoplasm. One anticipated problem that could occur with this procedure is keeping the cells alive, because transfecting the cells on a slide can be difficult to do (in comparison to the usual transfection in cell culture plates) without killing them. Trying different amounts of transfection quantities allowed the most appropriate amounts to be used in order to provide optimum results. Immunocytochemistry was also used to look at the interactions between ApoE4 and Sirtuins in the cell lines: A172 and SH-SY5Y. The same method noted above was used, but instead looked at whether ApoE and SirT1 interact and where in the cell this interaction occurs if it in fact does.

*Cell Transfection:*

A172 cells express APP endogenously, so they were transfected with the ApoE expression constructs. The SH-SY5Y cell line does not have either APP or ApoE and was transfected with both. Liposomes containing the desired protein were made using lipofectamine to introduce the proteins to the cells.

*Cellular Fractionation:*
In order to perform cellular fractionation, a kit from Thermo scientific was used. After harvesting the cells into the appropriate centrifuge tubes, one being a control of A172 cells that have not had ApoE4 transfected and one that has had APOE4 transfected. The appropriate reagents were added and spun down in the centrifuge at the appropriate speeds. After each spin the supernatant was removed and kept in the appropriate tube that pertains to that fraction. After the fractionation there was two fractions: the cytoplasmic extract and the nuclear extract. When doing this with the SH-SY5Y cells there was a control, SH-SY5y cells transfected with ApoE, and SH-SY5Y cells transfected with APP. After doing the fractionation for each different cell line, a western blot was ran and the membranes were probed for ApoE and APP to see in which fractionation these are both found. This method was used to show where in the cell ApoE4 is localized.

**Co-Immunoprecipitation:**

In order to look at the interaction between sirtuins and ApoE4 co-immunoprecipitation was used. This is the precipitation of a protein antigen using an antibody. Using the A172 cells and the SH-SY5Y cells, immunoprecipitation involves using beads that contain the primary antibody we are looking for. These beads allow for a high binding capacity. The wash steps and centrifugation allowed for the lysates to be separated, yielding our target protein more specifically. The precipitated protein was then examined using western blotting and examined what other protein interacts with it. This method gave us the data to see whether these two proteins do interact with each other

**Western Blotting:**
Using SDS-page, the presence of ApoE, APP, and Sirtuins was determined in A172 and SH-SY5y cell lines. Western blotting was used after cell fractionation to show what part of the cell these proteins are localized in and also after Co-Ip in order to see the interaction between ApoE and sirtuins. The blots were probed with antibodies that are epitope-specific for ApoE, APP, and SirT1 and 2.

*AlphaLISA:*

This technique allowed for the use of cell cultures to measure sAPPα levels and Aβ levels before and after drug testing. sAPPα and Aβ were standardized to produce a ratio between the two.

*SirT1 Assay:*

This kit was used on cells that were treated with a variety of different drugs. By adding a substrate and developer, a plate reader gave the calculations of the amount of SirT1 in each well.
References:


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