Investigations of Alzheimer's Disease: *Mechanisms of Assessing APOE 3 and 4*

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USE OF AI

AI, specifically ChatGPT and Perplexity, was used while writing this paper to assist me in the writing process. I used AI to generate initial ideas for the experimental design for future experiments, to help me organize the paper's structure according to the guidelines, to identify relevant scientific articles to use as references, and to proofread my paper for grammar mistakes and clarity.

Alzheimer's disease (AD) is a devastating neurodegenerative disease that affects millions worldwide, with no cure and limited treatment options. One of the most significant genetic risk factors for AD is the apolipoprotein E ε4 allele (APOE4). While we know that APOE4 increases risk for disease, the mechanisms by which it promotes neurodegeneration still remain unclear. Previous studies suggest that APOE4 can disrupt lipid metabolism, lead to synapse loss, and trigger inflammation in glial cells in the brain. These effects are often studied alongside other AD-related mutations, making it hard to determine which are caused directly by APOE4. The aim of this proposal is to isolate the effects of APOE4 by comparing cognitive and molecular changes in humanized APOE3 and APOE4 knock-in mice. Behavioral testing and gene expression analysis will be used to identify early-stage AD effects caused solely by APOE4. Understanding these mechanisms could offer valuable insight into AD progression and potentially help develop new strategies for early intervention in individuals who carry the APOE4 allele.

THE PHENOTYPE

Try to imagine losing track of a conversation, forgetting the faces and names of family and friends, or walking into your kitchen only to wonder why you're there. For many individuals with AD, these moments of forgetfulness are only the beginning of the disease. What might seem like ordinary brain fog can quickly evolve into the loss of independence and identity. AD is a progressive and degenerative brain disorder that primarily affects memory, thinking, and behavior. Over time, it destroys a person's ability to communicate, function, and recognize loved ones. In its final stages, AD often leaves individuals completely dependent on caregivers, as they are unable to carry out even basic tasks like dressing, eating, or speaking. For their families, the emotional burden can be overwhelming, and the sense of watching your loved one slowly fade away is devastating. While most people associate AD with the natural aging process, science has revealed that genetics can play a powerful role, especially in determining who is at risk of developing the disease and when. Among the many genes linked to AD, one stands out as possibly the most influential: *APOE*.

The *APOE* gene, short for apolipoprotein E, codes for a protein that helps transport cholesterol and other fats through the bloodstream. Its function is important throughout the body, but in the brain, *APOE* has additional important roles. It helps maintain neuron health, supports the repair of damaged brain tissue, helps regulate the blood-brain barrier, and can assist in clearing waste products, such as amyloid beta, the protein that clumps together to form plaques, a hallmark of AD.

However, not all *APOE* genes are created equal. There are three main alleles: ε2, ε3, and ε4. Every individual inherits two copies of the gene, one copy from each parent. The ε3 allele (*APOE3*) is the most common and is considered "neutral" for AD risk, as it doesn't significantly increase or decrease the risk of developing the disease. The ε2 allele (*APOE2*) is relatively rare but offers some protection against developing the disease. The ε4 allele (*APOE4*), however, is associated with an increased risk of AD and earlier onset of symptoms (Liu et al., 2013).

Previous research has shown that individuals with one copy of *APOE4* are about two to three times more likely to develop AD, and those who have two copies, homozygous carriers, are up to 12 times more likely to develop AD (Kloske & Wilcock, 2020). Research has found that more than half of all people with late-onset AD carry at least one *APOE4* allele (Mahley, 2016). Even more concerning is that research has shown that carriers of *APOE4* often experience brain changes decades before symptoms begin, such as reduced brain metabolism in memory-related areas like the hippocampus and increased inflammation and oxidative stress (Liao et al., 2017).

The symptoms of AD often begin subtly. An individual might start by misplacing items, forgetting names, or repeating questions. Over time, this progresses into deeper confusion, difficulty organizing thoughts, and eventually an inability to perform everyday tasks. Their language skills often decline, and emotional regulation can become impaired. In the late stages of AD, individuals may also lose awareness of the time, where they are, and even their own identity. It is important to note that not all people with AD progress at the same rate; carriers of *APOE4* tend to decline more rapidly, with earlier onset and more noticeable cognitive and behavioral symptoms (Neu et al., 2017).

Although *APOE4* can increase the risk of developing AD, it's important to understand that carrying *APOE4* is not a guarantee of developing AD. Some people with the allele never develop the disease, and some who do develop AD do not carry *APOE4* at all. This elevated risk provides a valuable opportunity for research. If scientists can understand what makes *APOE4* so damaging to the brain, they may be able to intervene before symptoms even appear. Catching the disease before widespread neuronal loss and its hallmarks are present could dramatically improve the chances of successful treatment or even prevention.

APOE4's impact is not limited to AD. It is also linked with worse outcomes after traumatic brain injuries (TBI), increased cardiovascular disease risk, and differences in response to some infections and medications (Tai et al., 2016). On the other hand, *APOE2* is protective against AD but is linked to a rare disorder called Type III hyperlipoproteinemia. These broader connections make *APOE* not just a gene of interest for AD, but also a central player in the health of the brain and body.

Because *APOE4* can affect so many processes in the body, from cholesterol transport to inflammation and neuronal repair, it provides a good target for studying how genetic risk can lead to biological damage. In my opinion, what makes this gene particularly interesting is its dual identity. *APOE* performs essential housekeeping functions in the brain but also contributes to its breakdown in the context of disease. My proposed experiment focuses on this, aiming to understand how *APOE4* influences brain cell function and gene activity before the onset of major disease symptoms. By doing so, the hope is to uncover molecular changes that could be reversed or slowed, potentially leading to new treatment strategies.

MOLECULAR FUNCTION OF THE GENE PRODUCT(S) AND MOUSE MODEL

The *APOE* gene, located on chromosome 19, encodes apolipoprotein E, a protein that plays an important role in the body's lipid metabolism, particularly cholesterol. In the brain, this protein has additional important functions. Unlike in the rest of the body, where *APOE* is primarily produced in the liver, the brain relies on local *APOE* production, especially by glial cells such as microglia and astrocytes. These cells produce *APOE* to help transport cholesterol and lipids to neurons, which need these fats for membrane repair, synapse formation,

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and normal signaling (Mahley, 2016). *APOE* also assists in the removal of waste products, including amyloid beta, a sticky protein that clumps into plaques in the brains of individuals with AD (Liu et al., 2013).

There are three major forms or alleles of the *APOE* protein: *APOE2, APOE3, and APOE4*. These alleles differ by just one nucleotide at two SNPs, rs7412 and rs429358, in exon 4, leading to a change of one or two amino acids. These small changes have a profound effect on how the protein folds and functions. *APOE3*, the most common allele, is considered "neutral," while *APOE2* offers protection against AD. *APOE4*, however, behaves quite differently. Its altered structure causes it to fold less efficiently, making it more prone to aggregation and misfolding. These changes disrupt its lipid transport and impair many of its normal functions. As a result, *APOE4* is less effective at clearing amyloid beta, contributes to synaptic loss, and triggers inflammatory responses, particularly by activating microglia (Mahley, 2016; Zhao et al., 2020).

The effects of *APOE4* on Alzheimer's disease are complex, as they vary depending on the context. In studies where *APOE4* is present alongside other AD-related mutations, such as those in the *APP*, amyloid precursor protein, or tau genes, it often amplifies the severity of the disease by increasing plaque accumulation and worsening cognitive decline (Shi et al., 2017). However, to truly understand what *APOE4* does on its own, researchers have created more refined models. One of these models includes humanized knock-in mice, in which the mouse *APOE* gene is replaced with the human *APOE3* or *APOE4* allele. These mice are generated using the CRISPR/Cas9 system or homologous recombination and are designed so that the human gene is expressed under the control of the mouse's regulatory elements (Foley et al., 2022). This avoids overexpression and allows for a more accurate representation of how *APOE* behaves in a real biological setting.

It's important to note that these knock-in models do not carry additional disease-causing mutations, unlike those found in *APP* or *PSEN1* transgenic mice. This makes them ideal for studying the allele-specific effects of *APOE*. Research using these mice has revealed that *APOE4* leads to early impairment of synaptic structure and plasticity, particularly in the hippocampus, which is important for learning and memory (Knoferle et al., 2014). Even in the absence of visible amyloid beta plaques, *APOE4* mice have shown deficits in behavior and cognition, as well as signs of microglial activation and oxidative stress. These findings suggest that *APOE4* may initiate the neurodegenerative process well before the pathological hallmarks of AD are detectable.

Previous studies have also used transcriptomics to examine how individual cells, specifically iPSCs, respond to *APOE4* at the level of gene expression. For example, using single-nucleus RNA sequencing (snRNA-seq), scientists have identified distinct gene expression patterns in iPSCs carrying *APOE4* compared to those carrying *APOE3*. These studies have shown altered expression of genes involved in actin filaments, regulation of epithelial-to-mesenchymal transition, axonal guidance, the endoplasmic reticulum stress pathway, and the innate inflammatory response (Zhao et al., 2020). This suggests that *APOE4* does not merely impair brain function; it can also alter gene expression in harmful ways.

Despite previous research on *APOE*, significant knowledge gaps remain. Many past studies focus on older animals or examine *APOE4* only in combination with other AD-related mutations. This makes it difficult to know whether observed effects are directly caused by *APOE4* or by a combination of various factors. Additionally, while inflammation and synaptic loss are frequently observed in older *APOE4* mice, little is known about the early changes that may occur in younger mice. Understanding these early changes is important, as it could provide opportunities for earlier therapeutic intervention.

This is what my proposed experiment will attempt to address. By using young humanized *APOE* knock-in mice without other AD-related mutations and pairing behavioral testing with cell-type-specific gene expression analysis, I aim to identify the earliest changes *APOE4* causes in the brain. Identifying these changes before major symptoms or irreversible damage occur could provide an opportunity for slowing or completely stopping AD progression.

EXPERIMENT FOR THE FUTURE

This research aims to determine how the *APOE* ε4 allele (*APOE4*) contributes to neurodegeneration by focusing on early synaptic dysfunction and neuroinflammation. Although *APOE4* is a well-established genetic risk factor for AD, the mechanisms by which it promotes disease progression remain unclear. To address this, I propose two experiments using mice that express human *APOE3* or *APOE4* alleles: one to assess behavioral and histological changes in memory-related brain regions, and another to examine cell-type-specific gene expression. I hypothesize that *APOE4* will disrupt synaptic signaling and promote inflammation prior to clinical symptoms, thereby identifying early changes that could be targeted for intervention.

The first experiment will evaluate whether *APOE4* expression alone is sufficient to affect cognitive performance and brain function in aging mice, independent of other AD-related mutations. Most previous studies have examined *APOE4* in combination with other genes linked to AD, such as the amyloid precursor protein (APP), making it harder to observe the individual contribution of *APOE4* to the early stages of the disease (Youmans et al., 2012; Shi et al., 2017). To better understand the impact of *APOE4*, I will use the CRISPR/Cas9 system to create genetically modified mice that express either the human *APOE3* or *APOE4* allele at the mouse *APOE* locus. These mice will still maintain regulation of *APOE* expression and are free of additional AD-related mutations, making it easier to isolate the effects of *APOE* gene alleles on cognitive health.

Behavioral testing in this experiment will be done on both male and female mice at two different ages: six months and twelve months. I chose these time points to capture the earlier stages of aging and any brain changes that *APOE4* might cause. The mice will undergo several behavioral tests, including the Morris water maze to assess spatial learning and memory, the novel object recognition test to assess recognition memory, and the open field test to assess exploratory behavior and anxiety (Knoferle et al., 2014). After behavioral testing, the mice's brains will be harvested for histological analysis. Immunofluorescent staining for synaptic markers such as PSD-95 (postsynaptic density protein 95) will be performed in regions of the brain critical for learning and memory, and particularly vulnerable in AD (Liao et al., 2017). The purpose of this first experiment is to determine whether APOE4 expression alone can cause measurable cognitive and synaptic problems before other features of AD, such as amyloid plaques or tau tangles, appear.

The second experiment will use single-nucleus RNA sequencing (snRNA-seq) to examine gene expression changes across multiple brain cell types in *APOE3* and *APOE4* mice. This will allow for examining how different cell types, such as neurons, glial cells, and immune cells, are affected by the presence of *APOE4*. This approach was chosen over single-cell RNA sequencing (scRNA-seq) because brain tissue can be difficult to dissociate into single cells, and isolating cells for scRNA-seq can trigger stress responses, altering gene expression. The nuclei for sequencing will be isolated from frozen hippocampal and cortical tissue collected from six-month-old *APOE3* and *APOE4* mice. These nuclei will then be analyzed to measure gene expression that may be altered by *APOE4*.

Previous studies have shown that *APOE4* affects microglial activation, reduces the expression of synaptic plasticity-related genes, and triggers inflammation in supporting brain cells, such as astrocytes (Shi et al., 2017; Zhao et al., 2020). However, these studies often focus on later stages of AD or use mouse models that are genetically modified in multiple ways. By focusing on middle-aged mice, this experiment aims to uncover the earliest molecular changes specifically linked to *APOE4* expression, before the onset of other disease markers. The results of this experiment will be analyzed to identify genes that are up- or downregulated in *APOE4* mice, as

well as any biological processes that may be affected by *APOE4*. I expect that *APOE4* mice will show upregulation of genes involved in immune activation alongside downregulation of genes involved in neuronal plasticity.

To ensure the results from both experiments are reliable, appropriate controls will be used throughout. *APOE3* mice will serve as the main comparison group to *APOE4*, as this allele is considered neutral with respect to AD risk (Liu et al., 2013). Regular lab mice (wild-type C57BL/6 mice) will also be included in molecular analyses to provide baseline data on *APOE* function. Due to some studies observing differences in *APOE*-related pathology between sexes, particularly with *APOE4* posing a higher risk to women in some human populations (Neu et al., 2017), both male and female mice will be analyzed in equal numbers. For the snRNA-seq, controls will include removing low-quality samples, filtering out data containing more than one nucleus, and normalizing gene counts to ensure consistency across all samples.

If my hypothesis is correct, I expect that *APOE4* mice will show behavioral impairments and reduced synaptic marker expression compared with *APOE3* mice. This would suggest that *APOE4* disrupts neuronal signaling even in the absence of amyloid pathology, aligning with the idea of a "preclinical" stage of AD driven by genetic risk factors. At the molecular level, I anticipate that snRNA-seq will reveal clear differences in gene expression between *APOE3* and *APOE4* mice. In *APOE4* mice, microglia will likely show a shift toward increased inflammation, while neurons may show decreased expression of genes critical for synaptic maintenance. These findings would provide strong evidence that *APOE4* causes early changes in the brain that could later lead to AD, highlighting potential targets for early intervention.

On the other hand, if *APOE4* mice do not show any significant behavioral or molecular differences from *APOE3* mice at the two examined ages, it would suggest that *APOE4's* effects occur later in life or that environmental and genetic stressors may be required to fully trigger phenotypes associated with *APOE4*. This wouldn't mean that *APOE* isn't important, but rather underscores its role as a risk amplifier whose effects may depend on age, stress, poor diet, or other variables to fully trigger disease processes.

There are several challenges to keep in mind when designing and interpreting these experiments. First, the effects of the *APOE4* gene may emerge slowly, requiring longer-term aging studies or the introduction of stressors, such as high-fat diets or low levels of inflammation, to reveal them (Tai et al., 2016). Second, although snRNA-seq provides insight into gene expression across different brain cells, it also generates a very large amount of data. Going through all the data will require a lot of time and computer programs to aid in analysis. This can be managed by working with researchers who specialize in bioinformatics and by using methods like DESeq2 to ensure results are accurate and meaningful. Finally, it's important to note that this research project focuses on the effects of *APOE* in a simplified genetic model. In the real world, AD risk involves complex interactions between many genes and environmental factors. Future studies could build on this approach to include other risk genes, such as *TREM2*, or examine how *APOE4* affects brain health when the body is dealing with other problems.

Together, both experiments provide a focused and realistic approach to uncover the early impact of *APOE4* on brain function. By combining behavioral testing, synaptic imaging, and single-nucleus RNA sequencing, this research project aims to determine how *APOE4* influences brain aging before visible signs of Alzheimer's disease appear. These findings have the potential to identify new therapeutic targets that could delay or prevent disease onset in genetically at-risk individuals, supporting the broader goal of developing precision medicine for individuals who carry genetic risk factors such as *APOE4*.

CONCLUSION

Understanding the molecular and cellular effects of the *APOE4* allele is essential for uncovering how *APOE* causes Alzheimer's disease. This grant proposal focuses on evaluating early behavioral, cognitive, and molecular changes in humanized *APOE3* and *APOE4* knock-in mice, specifically in the absence of additional AD-related mutations. By also analyzing brain tissue from mice and profiling gene expression in different brain cell types, I aim to pinpoint how *APOE4* influences neuronal health and immune responses in the brain, long before plaques or other hallmarks of the disease appear. This focus on early-stage effects of *APOE* fills an important gap in knowledge, as much of the existing research has focused on later stages of disease or used models that introduce multiple mutations at once.

If successful, this research proposal could reveal the earliest brain changes associated with *APOE4* and identify molecular pathways that could serve as ideal targets for early intervention. These findings could also help inform drug development aimed at inflammation, supporting synaptic function, or targeting *APOE*-related signaling before irreversible damage occurs. In conclusion, this research proposal aims to contribute to the growing field of knowledge on neurodegenerative disease by providing insight into how individual genetic differences can shape disease progression and potentially offer new treatment strategies.

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