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The use of CRISPR to prevent, or slow the progression of Alzheimer's disease.

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Abstract
Alzheimer's disease (AD) is an incurable disease that affects individuals over the age of 65. Medical treatments have been developed to treat symptoms, but they have not been sufficient. A number of hypotheses have been proposed about the (multifactorial) causes of AD, although the overwhelming focus has been on the β-amyloid hypothesis, which posits that abnormal accumulation and deposition of oligomeric or fibrillar amyloid β (Aβ) peptide in the brain triggers neurodegenerative processes. Genome wide association studies have identified multiple genes; with differing effect sizes; that may be associated with the onset and progression of cognitive decline; a hallmark of AD. By knocking in, or knocking out candidate genes in-vitro or in animal models, CRISPR has the potential to definitively assign causation to gain of function, or loss of function, genetic mutation(s), regardless of the presence (or absence) of specific biomarkers (such as fibrillar amyloid β (Aβ) peptide). CRISPR's biomarker agnostic potential can identify (and reduce) observational bias, false positives, and legacy false assumptions. These uses, in themselves, make CRISPR a game-changing technology in designing and advancing small molecule or protein drugs to treat neurodegenerative diseases. In addition, scientists have been trying to deliver CRISPR into the brain as a means to try to help prevent or slow the progression of the disease, with promising results in both in-vivo and in-vitro studies. Other neurological diseases, such as Huntington's disease and Parkinson's disease, have used CRISPR to treat the illness in mice and animal models. In the short term, CRISPR is an invaluable tool to correctly identify genes and biomarkers predisposing to the development of AD. In the long term, subject to drug-delivery advances and more efficient and precise genome integration, CRISPR-Cas9 may well find its way into the therapeutic armamentarium to cure AD.

Keywords
Alzheimer's disease, CRISPR-Cas9, β-amyloid, Plaque, Gene editing, Genome wide association studies, Biomarker, Clinical trial, Neurodegenerative disease, Tau protein

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Introduction

It is estimated that 1 in 85 persons worldwide suffer from Alzheimer's disease (AD). In 2015 the cost to care for persons with dementia was ~ $818 billion or ~ 1% of global GDP (1). This number will continue to increase if a cure is not found. After two decades of clinical trial failures, a change in trajectory with regard to AD mechanisms, hypotheses and drug design seems overdue.

AD occurs when an individual starts to lose their memory and is followed by severe confusion. A hundred years have passed since the first report of AD, and although medications such as acetylcholinesterase (AChE) inhibitors and memantine have been given to patients to alleviate symptoms, no cure has yet been found (2). Aducanumab is the only disease-modifying medicine that alleviates the symptoms of AD. This medicine is a human antibody that targets the β-amyloid protein and reduces amyloid deposits. Clinical studies have only been performed on people with early stages of AD and mild cognitive impairment (2). Studies are ongoing to determine whether or not this medication affects a person's rate of cognitive decline over time. Scientists are also attempting to use gene editing technologies such as CRISPR (C*lustered Regularly Interspaced Short Palindrome Repeats)* to help prevent or slow the development of AD. The results of both, in-vitro and animal in-vivo experiments appear promising. It appears that the use of CRISPR technology may prevent or slow the progression of AD. However, many unanswered questions remain, regarding safety, mechanisms, drug delivery, inadvertant or misplaced off-target recombination and long-term adverse genetic reactions.

Alzheimer's disease

AD occurs when brain cells degenerate and die, thereby causing loss of memory and extreme confusion. AD is caused by the abnormal build-up of proteins in the brain (2). As shown in Figure 1, one of the proteins involved is termed the β-amyloid protein, which forms plaques in the brain and is surrounded by dysfunctional neurons. The other protein is called tau; tau forms tangled deposits around the neurons, causing the start of neurodegeneration. These neurofibrillary tangles (NFTs) are composed of bundles of hyperphosphorylated tau proteins. When tau starts to hyperphosphorylate (loss of normal function and aggregates to form neurofibrillary tangles) it begins to disassociate from microtubules and starts to associate into NFTs, which in turn, initiate the neural damage (3). Excess glial cell activation may be another causative factor. Glial cells help protect the brain's neurons by maintaining homeostasis, cleaning up debris, and forming myelin. The myelin sheath can be described as an 'insulation' layer that forms around the neurons in the brain and spinal cord (4). The destruction of glial cells results in the loss of memory or an impairment in learning. β-amyloid or tau NFTs accumulate in the brain over many years, before symptoms start to manifest. Another indicator of AD is when certain areas of the brain start to decrease in volume, which may be another causative factor for the loss of memory (5). Initial symptoms may also include loss of vision or linguistic abnormalities. AD is most common among patients who are 65 years or older. About 1 in 14 people over the age of 65 and 1 in 6 over the age of 80 suffer from AD or from some form of dementia. About 1 in 20 individuals under the age of 65 report suffering from symptoms that could be indicative of AD;

this is the so-called early or young-onset AD (2). Other increased risks of AD include family history, Down's syndrome, severe head injuries, cardiovascular disease, hearing loss, depression, loneliness, and a sedentary lifestyle.

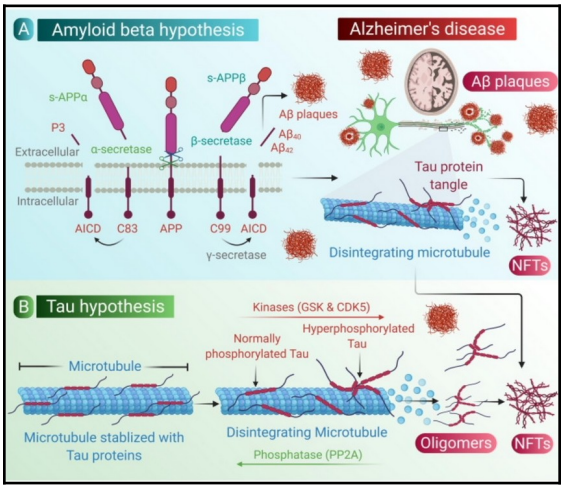

Figure 1. Process of Alzheimer's disease development in the brain (5). The activation process of β-amyloid and tau in the brain that leads to Alzheimer's disease.

Currently, there is no treatment for AD, but medicines can alleviate some of the symptoms. These palliative treatments include (AChE) inhibitors, memantine, and others to treat debilitative behaviors such as increased agitation, anxiety, wandering, aggression, delusions, and hallucinations (1). Other treatment methods include Cognitive Stimulation therapy, Cognitive Rehabilitation, and the reminiscence of one's life with the use of family pictures and favorite music. Lifestyle changes that may help prevent the development of AD include adopting a healthy non-sedentary lifestyle and staying mentally and socially active. There are multiple genes and loci that have been identified using Genome-Wide Association Studies (GWAS), associated with AD (6-7). Although the β-amyloid and/or tau hypotheses have been unequivocally and relentlessly pursued over the past two decades, several other mechanisms and hypotheses are also logically sound and merit perusal. Several of these hypotheses or mechanisms involve genes that are mutated and/or otherwise dysfunctional. The relatively recent methodology of gene editing, CRISPR, can potentially be used to a] determine the effect size of any individual gene, or of multiple genes, on the symptoms of cognitive decline, and b] to edit these genes, so as to prevent or slow the progression of AD.

The rate of amyloid removal in the context of clinical trials

Although drugs that target β-amyloid plaques in the brain have largely proven to not be effective, four anti-Aβ antibodies were shown to decrease plaque levels from the brains of patients with AD (8). One of these antibodies; Aducanumab, significantly reduced amyloid

plaques from the brain (8). This drug has been approved by the FDA but its benefits are still being debated. It is hypothesized that, not only the extent; but also the rate of plaque removal is important in order to observe clinical benefit in enrolled patients. Since Aβ pathology drives tau pathology, it may be that amyloid plaque needs to be reduced to a low enough level to reveal any significant benefit in the brain. The speed with which the amyloid level is decreased may hence be important so as to be able to observe a measurable clinical benefit in the clinical trial time-frame. Whereas the proposal certainly merits perusal, it could also be construed as yet another attempt – conceptually similar to the proposed *lack* of *soluble* β-amyloid as being contributory to AD - to salvage the β-amyloid hypothesis, such that research into other causes of AD is stymied.

Potential causes of Alzheimer's disease

Biomolecular condensates

The phenomenon of liquid-liquid phase separation (LLPS) has been invoked to describe the plaque formation in AD. LLPS is postulated to occur when susceptible RNA or polymeric molecules bind non-covalently with the intrinsically disordered regions (IDRs') of specific binding proteins (9). Any perturbation in the thermodynamics of this solute-rich, liquid phase transition can lead to irreversible precipitation as a solid phase or phase-separation into a solute enriched liquid phase.Tau is an important protein in the development of AD. Tau develops from a viscous gel phase into anomalous protein aggregated tangles, in a case of LLPS gone awry. It then becomes thioflavin-S positive suggesting that the β-pleated sheet present in tau forms in-vivo (10). The separation of tau can be catalyzed by electrostatic interactions in the unstructured N-terminal half of tau together with the hydrophobic interactions at the C-terminal which stabilize the tau droplets toward the β-sheet. Protein aggregation may hence proceed via LLPS and progress to neuronal disease. Mutations in the IDRs' - but elsewhere as well - of FUS, p62, TDP-43, hnRNPA1, TIA1 and a host of other proteins and chaperones involved in autophagy, RNA binding and lysosomal acidification increase the ability of phase transitioning the otherwise reversible LLPS phenomenon into solid plaque fibrils or tangles. CRISPR can be used to prevent the formation of these tangles by editing the relevant mutated gene(s).

Cholinergic Hypothesis

The cholinergic hypothesis states that the dysfunction of acetylcholine containing neurons, contributes to cognitive decline and the development of AD (11). The results of both post mortem and antemortem studies in aged humans and AD patients, as well as animal experiments suggest that a host of cholinergic abnormalities including alterations in choline transport, acetylcholine release, nicotinic and muscarinic receptor expression, neurotrophin support, and perhaps axonal transport may all contribute to cognitive abnormalities in aging and AD. Cholinergic abnormalities may also contribute to the deposition of toxic neuritic plaques in AD (12). Currently available FDA-approved cholinesterase inhibitors for the treatment of AD include donepezil, rivastigmine, and galantamine.

Autophagy & Autosomal Acidification

Autophagy is the process of lysosomal degradation which maintains cellular

homeostasis and removes non-useful proteins and organelles from the cell. In the autophagy-lysosomal pathway (ALP) a double membrane surrounds the cytoplasm by an adaptor protein and occupies specific target substrates that are close to forming an autophagosome (APs). They develop into autolysosomes (ALs) when they fuse with endolysosomes (LY) which introduce acidic hydrolysis and vATPase. LYs are an important cause of the formation of the gene that initiates AD and the build-up of amyloid precursor protein (APP) (13). AD is defined by two lesions: intracellular tau aggregates and neuritic plaques that consist of dystrophic neurites (DNs). It is difficult to determine autophagy dysfunction in AD and the production of APP because it is challenging to monitor ALP abnormalities in-vivo in the brain. To overcome this difficulty, transgenic mice (TRGL) were used with an autophagy adapter protein associated with AP and AL (13). The TRGL and AD mice models showed evidence of lysosomal vATPase activity, autophagy dysfunction in some neural areas, APP-βCTF, and β-amyloid with poorly acidified AL before β-amyloid deposition. Furthermore, the autophagic stress response showed neurons being packed with Aβ/APP-βCTF-filled Aβ-positive autophagic vacuoles (AVs). The fluorescent petal-like blebs that surrounded nuclei of neurons generated flower-like profiles called PANTHOS. The combining of AV with the endoplasmic reticulum, generates the production of β-amyloid plaques in the brain. To further observe the AL acidification in the brain of AD mice, researchers isolated AL/LY-rich fractions by density centrifugation and monitored their vATPase activity. The vATPase activity in AL/LY of 6-month-old AD mice was decreased compared to the aged wild-type littermates; showing a further decline after 12 months.

Blood Brain Barrier breakdown as an indicator of cognitive dysfunction

Small vessel disease of the brain has been estimated to contribute to approximately 50% of all dementias worldwide, including those caused by AD (14). Studies have shown that the formation of β-amyloid and tau occurs in conjunction with blood vessel abnormalities in the blood-brain barrier (BBB) (14). Individuals who showed signs of early cognitive dysfunction develop brain capillary damage and BBB breakdown in the hippocampus regardless of whether or not β-amyloid or tau are present (14). The breakdown of the BBB hence appears to be an indication of human cognitive dysfunction independent of the presence of β-amyloid and/or tau.

Lipid Metabolism and the Lipid-Chaperone hypothesis

Increased levels of fatty acids, acyl-carnitines, and acyl-CoA may be toxic to the brain and can cause mitochondrial uncoupling and bioenergetic dysfunction. Changes in the quantity of unsaturated fatty acids have been linked to the development of AD (15). Patients who suffer from AD have lower plasma levels of unsaturated fatty acids. In the hippocampus part of the brain, docosahexaenoic acid (DHA) levels are significantly lower in AD patients. Lipid rafts are cholesterol and sphingolipid-enhanced membrane structures where proteins of synaptic transmission and amyloidogenic secretases are located. The lipid rafts in AD brains have lower ω-3 PUFA and MUFA (primarily oleic acid) and lower unsaturation levels for the phospholipid acyl chain in the cortex. Research indicates that changes in lipid

rafts are the main cause of alteration in lipid homeostasis in the early stages of AD brains.

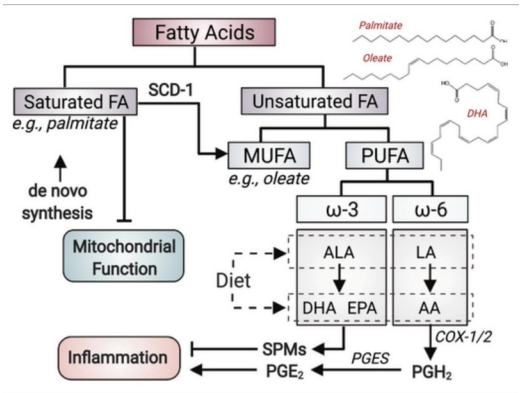

Figure 2: Fatty acid classification and effects (17).

Lipids may play a major role in the formation of amyloids in AD. Intrinsically disordered proteins (IDPs) misfold into dysfunctional shapes and collect together as amyloid aggregates when they interact with proteins and lipids membranes (16). Figure 2 shows several classifications of fatty acids. It is however not well understood how amyloidogenic proteins move from an aqueous environment to the hydrocarbon core of the membrane (17). The toxicity of amyloid proteins may be linked to their harmful effects on membrane integrity.

Implications of the plaques being granulomas

Granulomas are dense collections of mononuclear phagocytes, mostly macro phagocytes, that form due to unknown infections or foreign anatomical stimuli (18). These structures are common in the body when silica and other types of metals have been inhaled (schistosomiasis), and in atherosclerosis, when insoluble proteins or lipids become deposited in the arteries. Research suggests that β-amyloid plaques may form from these granulomas. Studies on mice demonstrate that these plaques are not formed spontaneously but are built by microglia (18), the most abundant tissue macrophage in the central nervous system. The microglial formation of dense-core plaques may be an attempt to decrease the widely disseminated neurotoxic Aβ oligomers and proto-fibrils by sequestering them into plaques. If dense-core plaques are indeed granulomas, their simple disassembly may not be necessary- or indeed even be contraindicated - as an Alzheimer's therapy.

Alzheimer's as an Autoimmune disease

Many researchers hypothesize that AD is an autoimmune disorder. Autoantibodies are the main facilitators of autoimmune diseases. Autoantibodies against β-amyloid, tau, microglia, and glial fibrillary acid protein have been observed in patients with AD (19). However, their role in disease pathophysiology remains uncertain. Amyloid-β, is itself, conceptualized as an immunomodulatory oligomerizing cytokine with antibacterial properties. It may be released as an early

responder cytokine in response to trauma, ischemia or infection. It is then postulated to attack "self" neurons, because of the electrophysiological similarities between neurons and bacteria. The subsequent breakdown products of necrotic neurons elicit further release of amyloid-β leading to a chronic, self-perpetuating cycle.

Infection Hypothesis

There are several receptors of β-amyloid, but the most common one is microglia (20). It engulfs the Aβ and destroys it. However, when too much Aβ is accumulated and starts to deposit in plaques, the microglia are unable to engulf all of the β-amyloid. The pathogen recognition receptors (PRR) such as TLR, CD36, and RAGE sense the presence of excess β-amyloids and, in turn, upregulate cytokine production (20). This initiates the destruction of neurons. This process causes the simultaneous hyperphosphorylation of the tau protein, which leads to the destruction of synapses and neuronal death. Itzhakai et.al. have hypothesized that microorganisms have a role in the development of AD (20). They showed that HSV-1 leads to the production of amyloid precursor protein (APP) which in turn leads to the excess development of Aβ and to the hyperphosphorylation of tau. Another study detected *Borrelia Burgdorferi* and *Chlamydophila pneumonia* in the post-mortem brains of patients who had suffered from AD (21). These pathogens manufactured biofilms to protect themselves from host defense mechanisms. These patients also had increased anti-C *pneumonia* present in their blood. *Staphylococcus aureus* was found in some Kupffer cells and became reactive under some circumstances (20). These infections led to the activation of the APOE4 gene that leads to the formation of AD. *Porphyromonas gingivalis* may be considered pathogen-associated molecular patterns (PAMPs) that interact with PRRs, which leads to cytokine production and interrupts the blood-brain barrier (BBB). This infection causes AD (21). *Porphyromonas gingivalis* results in the formation of other microorganisms in the brain that also aids in the development of AD. *Treponema pallidum* also leads to infections that can invade the brain and cause an infection resulting in AD (20). Several fungi, such as *Candida albicans and Malassezia,* have contributed to AD (21). These organisms are found in the post-mortem brain of patients who have had AD, and environmental interactions can impact the risk of the migration of these microorganisms past the BBB, to the brain.

Overall, there are several hypotheses proposed as being causative of AD. CRISPR, a gene editing tool, can potentially be used to edit candidate genes associated with the development and progression of the disease. There are ~ 50 genes associated with AD. The inducible pluripotent stem cell Neurodegeneration initiative (iNDI); an NIH funded program; utilized CRISPR to generate multiple Single Nucleotide Variants (SNV) per gene across several targets in iPSCs'. A standardized collection of isogenic iPSC lines carrying over 100 AD related mutations could hence be generated to enable large scale AD disease recapitulating and modeling.

CRISPR

Clustered regularly interspaced short palindrome repeats, or CRISPR, describes a segment of DNA containing repetitions of base sequences involved in the defense mechanisms of prokaryotic organisms or archaea (22).

CRISPR-Cas9 is an enzyme used to cut DNA, almost like a genetic scissor. CRISPR can precisely target, edit, modify, regulate, and mark genomic loci containing an abundance of cells and organisms. Scientists can now use this tool to edit the genomic sequence of interest in several organisms in-vitro or in-vivo. As depicted in figure 3, this process is called gene editing.

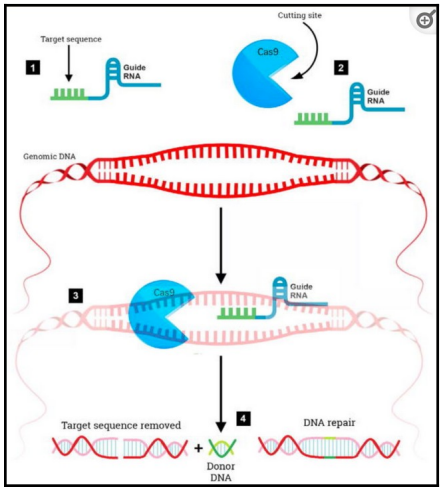

Figure 3: CRISPR-Cas9 mechanism of action (22).

CRISPR-Cas9 consists of two key molecules: a Cas9 enzyme and a guide RNA strand (23). The Cas9 enzyme is used to cut the DNA strands at a specific location in the genome. The guide RNA (gRNA) consists of small pieces of pre-designed RNA sequences that are about 20 bases long and are located within an RNA scaffold. The scaffold binds to the DNA, and the pre-designed sequence aids the Cas9 enzyme to move along the genome. This ensures that the Cas9 enzyme cuts at the correct location. The Cas9 enzyme then follows the guide RNA to the location of the DNA sequence and cuts across both strands of the DNA. The DNA repair machinery of the cell activates in an attempt to repair the break. Scientists can harness this natural DNA repair machinery to make changes to more than one gene in a particular cell. As shown in figure 4, there are two major ways that CRISPR can be used: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (24). The HDR repair technique uses donor DNA to precisely repair double-strand breaks (DSBs) for gene alteration with low efficiency. NHEJ usually results in gene alteration or deletion for gene distribution and higher efficacy. NHEJ is more error prone than HDR and can introduce random insertions and deletions at the DSB site. Cell division can occur by combining the techniques of gRNA and CRISPR. CRISPR-Cas9 is being tested to determine whether it can help with slowing or preventing the progression of neurological diseases (24).

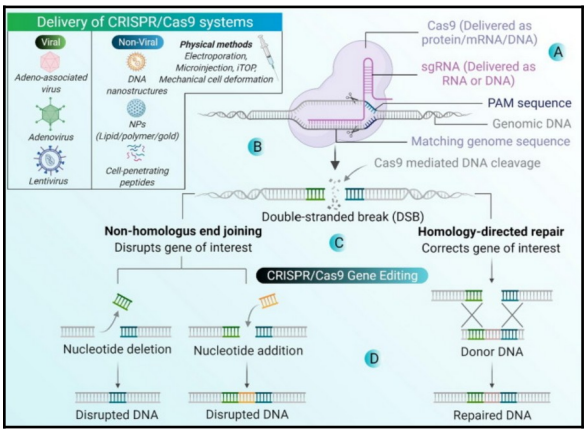

Figure 4: The difference between NHEJ and HDR in terms of the mechanics of gene editing using CRISPR-Cas9 (24).

CRISPR's potential to help prevent Alzheimer's disease in humans

AD leads to adverse health effects, imposes a socio-economic burden and cannot be prevented with current drugs (1). The question is: can CRISPR technology help prevent or slow the progression of AD? The simple answer is not yet, but there are promising studies that may create potential solutions in the future. CRISPR can aid in targeting the specific gene(s) that cause(s) AD and editing it, thus theoretically preventing the disease from occurring. This has to be done before symptoms arise.

The *raison d'etre* of CRISPR is to edit gene(s). Once the genes/loci, the effect size and their interactions have been identified as being AD causative – again; using CRISPR – editing those gene(s) using CRISPR – regardless of whether or not conventional wisdom defining symptoms have developed – should prevent or delay the progression of the disease. The CRISPR-Cas9 technology is not – and is not meant to be – palliative. Rather; it is meant to diagnose and prevent (disease onset or progression). Whether or not signaling pathways, events or biomarkers coalesce or manifest into downstream NFTs' or beta-amyloid plaques should have no bearing on editing upstream (CRISPR identified causative) gene(s) or loci. The history of AD drug development is littered with failed clinical trials. According to one analysis, "....Unfortunately, these clinical trials have failed resoundingly with more than 400 failed clinical trials since the last Alzheimer's drug-which only temporarily treats the symptoms of the disease-was approved more than a decade ago....." (23). CRISPR can correctly diagnose the effect size of individual genes thereby determining the magnitude of contribution of particular downstream biomarkers to symptom development and disease progression. The correct identification of causation predictive biomarkers can break the decades-old impasse in AD drug development and clinical trails,

enabling disease modifying drugs to be developed in years; rather than taking decades.

The β-amyloid hypothesis attributes AD to β-amyloid NFTs and hyperphosphorelated tau. Therapies in clinical trials attempt to decrease the rate of formation of these proteins, with the implicit assumption that these stuctures are the only (or the predominant) biomarkers of causative event(s). This assumption may be incorrect, as evidenced by case control participants who have negligible cognitive decline even in the presence of β-amyloid NFTs. CRISPR can screen for specific genes that cause cognitive decline regardless of whether those genes are necessary and/or sufficient for β-amyloid NFT formation. CRISPR hence is significantly advantageous in disentangling biomarker observational bias and clinical symptoms; not only in AD; but in other neurodegenerative diseases.

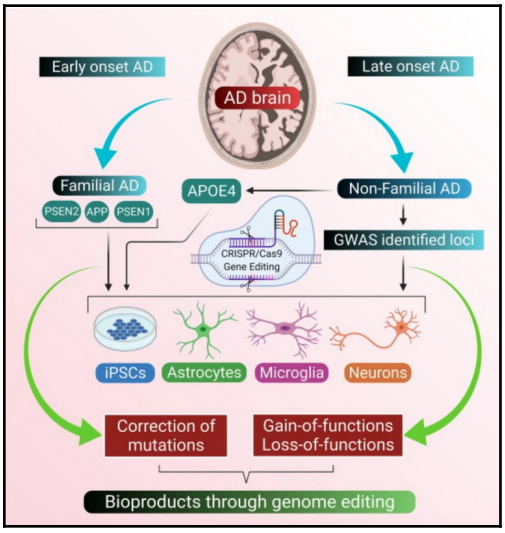

Figure 5: The different stages of Alzheimer's disease (25).

As presented in figure 5, there are usually three groups of patients who suffer from AD; the first group is called Early-Onset AD (EOAD), which occurs between the ages of 30 and 60 years, and Late-Onset AD (LOAD) or Sporadic AD (SAD) which occurs after the age of 60 (25). It has been shown that using CRISPR to treat SAD is not as effective. Familial AD (FAD) occurs in people usually between the ages of 40-60 (24). FAD, EOAD, and SAD all involve altered β-amyloid metabolism. Thus, treating β-amyloid is important regardless of whether CRISPR is used. EOAD is usually caused because of amyloid-β precursor protein (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2) mutations of the genes. LOAD is caused by a gene called Apolipoprotein E or APOE. If an individual contains an APOE on the E2 allele, this reduces their risk of having the disease by 40% (22). APOE3 is the most common gene and does not seem to produce any risks. However, APOE4 increases the risk for EOAD and is present in approximately 15% to 25% of individuals. Having one copy of

APOE3/APOE4 increases the risk of developing AD by 2 to 3 times and having two copies of APOE4 magnifies that risk 10-15 times (25). One potential use of CRISPR would be to change APOE4 to APOE2 or E3; this may help prevent AD from occurring (25). Along these lines, CRISPR has been used in trials to devise a cure for the APP Swedish mutation in mice and has produced promising results.

In-Vivo Results using CRISPR

CRISPR has been used to target the β-amyloid (Aβ) precursor protein (APP) gene mutation in mice (23). This mutation (K595N/M596L) located in exon 16 has been associated with increased Aβ production due to abnormal APP cleavage by β-secretase (Bace1), and causes familial AD. This leads to β-amyloid production and causes tau-hyperphosphorylation resulting in AD. Scientists injected DNA encoding information using CRISPR-Cas9 and guide RNAs in adeno-associated virus vectors (AAV) in the hippocampus of transgenic mice (24). However, only 2% of the transgenes were disrupted in the specific injected area. The authors found that since more than 100 copies of the transgene were present per neuron, the injected CRISPR-Cas9 was insufficient to correct the mutation. The editing efficiency of CRISPR-Cas9 hence needs to be increased so that a large number of transgenes can be edited using a limited number of CRISPR-Cas9 molecules. More research is also needed to determine if transgenic mice harbor a similar proportion of transgenes per neuron when compared with analogous mutations in humans.

In a different study in 2020, a mouse AD model was made to generate a β-amyloid sequence in their APP gene using CRISPR-Cas9. Researchers injected a Cas9-Bace1 nanocomplex in the hippocampal of a 6-month-old AD mouse (26). Within four weeks, a decrease in BACE1 and β-cleavage products of APP was found in the AD brain. The gathering of β-amyloid is an important factor that causes AD. β-secretase (Bace1) is important for the combination of β-amyloid peptides that target the Bace1 gene in the CA3 hippocampus of FAD-affected transgenic mice. In a series of experiments, Cas9-Bace1 (which is a nano complex-mediated CRISPR-Cas9 used to target genes and neurons in mouse models) reduces Bace1 and lowers Aβ1–42 secretions (26). Cas9-Bace1 significantly improved cognitive impairment in mice and targeted the genes and neurons that can help treat AD in other mouse models (26).

Different experiments have found that reducing the glia maturation factor in cells by using CRISPR-Cas9 helps regulate GMF-induced proinflammatory in microglia (27). Cysteinyl leukotrienes are also inflammatory lipid molecules and begin inflammatory signaling cascades by two major G-protein coupled receptors (CysLT1R and CysLT2R). Research shows that CysLT1R is associated with the development of AD. Increased levels of Aβ1–42 cause CysLT1R expression. Using CRISPR-Cas9 to delete CysLT1R was shown to reduce β-amyloid production and neuroinflammation in mice (28).

Furthermore, melatonin and melatonin receptors have helped decrease the progress of AD (29). Melatonin helps protect neural cells from the toxicity of β-amyloids. The activation of the Melatonin or Mt1 gene gland using CRISPR-Cas9 protects the neurons. This

experiment was done in-vivo by injecting the Mt1 gene into the neuronal cultures of the hippocampal and cortical regions of the mice's brains (30). After two weeks of monitoring, the Mt1 gene did help prevent the degeneration of neurons in AD. This experiment also demonstrated that the Aβ42/Aβ40 ratios were significantly reduced. Aβ42/Aβ40 are β-amyloids that have a 42/40 residue. The primary difference between the two is that Aβ42 has two extra residues on the C-terminus. Some experiments that were successful in mice have been unsuccessful in humans. For example, scientists treated mice with Aβ1–42 in order to lower the amount of β-amyloid in the brain (30). When this method was repeated in humans, they acted adversely to the reaction and developed meningoencephalitis. This was probably due to the adverse effects of the T-cells adapting to the large Aβ1–42 particles. Figure 6 shows an example of the difference between Aβ1–42 genes in humans and mice in terms of amino acid substitutions. Other trials using drugs such as bapineuzumab and solanezumab were successful in mice but not in humans (31). All in all, recent in-vivo experiments in mice have been promising for future studies involving the use of CRISPR to cure AD. However, it is known that there are many differences between mice and humans, and therefore alternative models are essential to understand in more depth the human-specific AD. With the ongoing studies in mice, there are key differences between mice and humans that need to be taken into consideration.

Figure 6: The difference between Aβ1–42 genes in humans and mice (30).

Differences between Mice and Humans

Mice have been very beneficial in studying human disease and mental disorders because they are mammals with short life spans and the protein-coding regions of the mouse and human genomes are 70 percent identical. However, there are several significant differences between mice and humans. The most apparent include anatomical as well as developmental timing differences. As regards the brain, the human brain is one of the most complex structures in biology. It contains 16 billion neurons and 61 billion non-neuronal cells (including glial, epithelial, pericytes, and endothelial) organized into 100 anatomical regions (31). The human cortex is 1000 times bigger in size than the mouse brain. The regulatory, structural, and functional programs differ between mice and humans. For example, motor neurons (MN), which are nerve cells that help in passing impulses along the brain and spinal cord to a specific muscle or body part, require about 3-4 days to develop in mice, whereas, in humans, they require about three weeks. As another example, the oscillatory gene which helps regulate rhythmic patterns in the vertebrae and aids in circadian rhythms, takes about 2-3 hours to develop in mice and 5-6 hours in humans. The physiological and anatomical developmental stages of mice and

humans are significantly different and may not be time normalizable in the context of drug development (32). Furthermore, gene expression programs, epigenetics, evolutionary trajectories and microbiomes are significantly different in mice and humans. Results of experiments in mice may hence not be directly extrapolable to humans (33). Alternative models for humans are necessary.

In-Vitro Experiments in 2D Cultures and 3D Brain Organoids

CRISPR testing in humans is fraught with ethical concerns and constraints. A way to circumvent this limitation is to perform in-vitro testing on human stem cells. Two-dimensional brain models of FAD and SAD with the use of Induced Pluripotent Stem Cells (iPSC) show similar features of AD pathophysiology such as the collection of β-amyloid and tau hyperphosphorylation (3). By using CRISPR-Cas9 in 2D cultures, the APOE4 gene can be altered to APOE2 or APOE3 to help prevent the effects of AD. APOE4 contributes to the pathology of tau phosphorylation in the brain (3). In an experiment with mice carrying the APP Swedish mutation, scientists used CRISPR-Cas9 to truncate the C-terminal region of the APP to prevent the interaction with Bace1, which abrogated the generation of β-amyloid (23). The experiment was performed in 2D cultures using human iPSCs and mouse brains and demonstrated that CRISPR treated APP effectively reduced the production of β-amyloid (3). However, the neurons derived from the iPSCs of embryonic stem cells (ESCs) have not been able to show morphological and functional properties of the human brain, but specific cells using 2D cultures can be studied to identify new samples of the neurodegenerative brain (34). The authors concluded that, "Neuronal maturation and development of synapse connections are governed by cell-cell and ligand-receptor signaling, which is not sufficiently established when neurons are cultured in a monolayer" (34). Cells growing in a single layer (monolayer) in a petri dish fail to show functional interactions and regulatory functions that are observed in the oligodendrocytes, astrocytes, and microglia of the human brain (35). Also, because the in-vivo process takes longer in humans, it is difficult to time-extrapolate conclusions from the monolayer cultures to human life spans. 2D cultures do not display many β-amyloid collections, and even when a large number of samples are taken from FAD genetic mutations, results are ambiguous. The authors further observed that "Moreover, the absence of interstitial compartment is believed to inhibit extracellular β-amyloid aggregation in 2D cultures" (34). Nonetheless, 2D models still have significant advantages as they are easy to manipulate, are less time-consuming, and can be made larger to scale for drug development. In addition, this more simplistic model can identify the most fundamental effects of the disease. This may allow researchers to identify novel techniques or treatments to combat the disease in-vivo.

While 2D cell cultures help detect the early stages of AD using iPSCs, scientists are now performing in-vitro experiments on three-dimensional (3D) brain organoids (generated by human iPSCs) to find more ways to detect the early development of the disease. 3D organoids are self-organized structures that are derived from stem cells and display morphological and functional similarities with several other intricate organs like the brain (3).

The 3D brain organoids are able to replicate certain parts of the brain thoroughly. They lack the cytoarchitectural organization to replicate the actual complexity that occurs in the brain (i.e., cell-to-cell interactions), but they are more complex than 2D models. They have been useful in studying AD and can allow us to learn more about human disease and any possible species-specific differences. In a recent study, scientists genetically overexpressed the APP and PSEN1 genes which led to the production of β-amyloid and tau (34). The 3D models showed that while carrying the FAD genetic mutation, there was an increased resistance to phosphorylated tau, indicating that we can learn from these more complex in-vitro models (36). In the future, CRISPR can be used with 3D brain organoids to aid in finding a cure for AD. CRISPR can be used to prevent tau collection by editing the gene to reduce its likelihood of forming clumps.

In the past ten years, numerous in-vitro models have been developed for AD. CRISPR-Cas9 could help develop AD models more efficiently. As an example, the down-regulation of Thioredoxin-interacting protein (Txnip) levels by CRISPR-Cas9 in HT22 murine cells reduced β-amyloid-induced protein cysteine oxidation modification (28), thereby identifying Txnip as a potential target for AD. Such in-vitro models, which can be either 2D or 3D, can also be developed using patient-specific iPSCs, which could aid in identifying the influence of different genomic profiles on specific biomarkers.In the future, utilizing human-specific AD models, that reflect human genetics and physiology, will help to investigate mechanisms of AD and other forms of dementia. Furthermore, by having human-specific AD models, researchers can explore whether some of the different hypotheses of the causes of AD are true and understand how they can develop AD, how it progresses, and understand the differences. However, not all hypotheses can be tested with in-vitro models since these models are restricted and do not recapitulate the whole body as well as in-vivo animal studies can. In-vivo studies are important and cannot be replaced only by in-vitro studies but can help add information gained by the in-vivo studies and potentially identify species-specific differences. 2D and 3D human-specific models can help identify possible biomarkers for detecting AD early and also do drug screens. In the future, the correlation between in-vivo and in-vitro data can help develop better techniques and ideas for curing AD.

Delivery of CRISPR-Cas9 to the Central Nervous System

There are many ways in which CRISPR-Cas9 can be delivered into the CNS. Previously CRISPR-Cas9 was delivered into the RNP (ribonucleoprotein) system using microinjection, electroporation, biolistic, and microfluidic techniques (37). However, these have strict requirements and are expensive. A study shows that CRISPR RNP electroporation of zygotes can increase embryo viability (37). A Lipid nanoparticle-based CRISPR RNP combined microfluidic could deliver Cas9 into the brain (37). Research has shown that the plasmid-based technique has a greater editing efficiency, but is linked to cell toxicity (37). RNA delivery also possesses these limitations of a high cell toxicity and low editing efficiency (37). However, the RNP-based delivery of CRISPR-Cas9 has a better balance

between cytotoxicity and editing efficiency (25). If CRISPR is not delivered into the CNS correctly, it can lead to other problems, such as off-target effects, including unintended point mutations, deletions, insertions, inversions, and translocations (38). New and advanced tools, such as CRISPR-P and CHOPCHOP, have been created to help prevent off-target effects while performing intricate alterations in DNA strands (38). Additionally, it is possible to control the amount of Cas9 injected into an mRNA or protein. Efforts to limit off-target effects have been very recent, and more testing is required to understand how these off-target effects occur and how they can be prevented. Nevertheless, it is imperative that CRISPR is delivered appropriately in the CNS else it could lead to many short – and long term – adverse effects.

CRISPR advancements in other Neurological Diseases

CRISPR has shown promising results in treating neurological diseases such as Huntington's disease in transgenic mice and has been able to test successful disease models for Parkinson's disease in Rhesus macaque. Huntington's disease is an inherited disorder in which the neurons in parts of the brain break down and die (39). This disease worsens over time and attacks motor control regions of the brain. Some symptoms of this disease include clumsiness and stumbling, difficulty concentrating, memory relapses, mood swings, and depression. This disease occurs between the ages of 30-50 and is currently incurable. However, clinical studies in mice have shown some success. Biswas et. al., reported that CRISPR successfully targeted mutant HTT (Huntington gene) in a mouse model of the disease (39). Using *Staphylococcus aureus* (SaCas9) allowed packing a single AAV vial for in-vivo delivery in the mouse striatum. SaCas9 pairing with RNA helped disrupt the mutation in-vivo and alleviated motor-deficiency symptoms. These results are promising and can be used as a template for using similar CRISPR techniques in humans to target dysfunctional AD genes.

In Parkinson's disease, dopamine producing neurons in the brain slowly break down and die. Some symptoms that may occur are an expressionless face, arms not swinging while walking, speech becoming soft or slurred, loss of smell, tremors in the limbs, and cognitive impairment (40). This disease usually occurs after the age of 60 and about 5% to 10% of the time after 50 (40). Currently, there is no cure for this disease. Carbidopa-Levodopa is used as a medication to help control symptoms. It allows dopamine to cross the BBB so that that its deficiency is alleviated. Scientists used CRISPR/Cas9 mediated PINK1 deletion to generate a Rhesus macaque model, recapitulating the human Parkinson's phenotype (40). They observed neuronal loss in the substantia nigra region in the midbrain of the affected animals. The CRISPR/Cas9 mosaicism enabled different degrees of PTEN-induced Putative Kinase 1 (PINK1) deletion, allowing researchers to understand the complexity of the associated phenotypes. A similar technique can potentially be used with AD by to create models that deposit different amounts of β-amyloid, and tau protein in the brain. Transgenic animal models have been created using CRISPR to study the progression of AD (30, 41, 42). Overall, CRISPR has shown encouraging results in mice models for Huntington's disease and Rhesus macaque models in Parkinson's disease. These

promising results and techniques could be applied to find a cure for AD. Table 1 summarizes several recent studies that were conducted in-vivo and in-vitro using CRISPR targeted genes or proteins. Some of these studies utilized CRISPR modified cells or animals as AD models.

Table 1: Selected recent studies for CRISPR-targeted genes/proteins and CRISPR-modified cells or animals used as models of AD. References are in parentheses in the first column.

Gene/Protein of interest	Cell line or animal model	Effect
PFN1, INPP5D (43)	Human iPSC line expressing induced-transcription factor microglia-like cells	Microglial phagocytosis modulators
MAPK14, CSF1R (44)	Human iPSC	Modulates SPP1 state of microglia
SPP1 (45)	Mouse model	Modulates synaptic phagocytosis
PSEN1 M146L mutation (46)	Human fibroblasts	Reducing plaque by reducing extracellular Aβ42/40 ratios
Mutated APP allele (47)	Transgenic mouse model	Reducing amyloid-β protein plaque formation
APP A673T mutation (48)	HEK293T cells	Reducing the cleavage of APP by β-secretase
DNA methylation APP (49)	Mouse model and mouse fibroblasts	Decreased amyloid-β (Aβ) peptide level and Aβ42/40 ratio
Combined targeting of master sites and p38α (50)	Transgenic mouse model	Block intrinsic augmenting mechanisms of tau hyperphosphorylation
PLCG2 P522R mutation (51)	Mutated microglia from Human STEM cells implanted into transgenic rodent models	Increased expression levels of several microglial genes that are reduced in people with AD
ANKLE2, BANF1, NUSAP1, EIF1AD, VPS18 (52)	Tau biosensor cells	Restrict tau aggregation initiated by both exosomal and vesicle-free tau seeds
AKAP9 rs144662445 mutation (53)	SH-SY5Y P301L neuron-like cells	Increased the level of phosphorylated tau
CHCHD6 (54)	HT-22 cells	Reduced CHCHD6 enhances APP accumulation
TTL (55)	Mouse model	Expression of TTL, by restoring microtubule entry into spines, suppresses the loss of synapses induced by amyloid-β peptide
NMNAT2 V98M and R232Q mutations (56)	Mouse model	Constitutive expression of SARM1, the central executioner of programmed axon degeneration
KIBRA rs28421695 mutation (57)	Mouse model	Regulates Aβ metabolism by controlling extracellular vesicle formation via the endosomal-lysosomal system
BRD2, FUS, TRIM28, PHOX2A, TSC2, VPS33A (58)	SH-SY5Y neuroblastoma cell line	Genome-wide CRISPR screen (GWCS)
TDP-43 (59)	SH-SY5Y neuroblastoma cell line, iPSC-MN cells	Loss of TDP-43 results in the inclusion of a cryptic exon in *UNC13A* mRNA and reduced UNC13A

		protein expression
IL1RL1 rs1921622 mutation (60)	hCMEC/D3 cells	Decreases sST2 levels, lower AD risk in females carrying the Apolipoprotein E (*APOE*)-ε4 genotype
TAGLN3 (61)	Human iPSC cell lines	APOE4 exerts a dominant negative effect to prime astrocytes toward a pro-inflammatory state that is pharmacologically reversible by TAGLN3 supplementation

Conclusion

AD, which affects many, is currently incurable. This may be because, even though historical and emerging evidence is suggestive of it being a multifactorial disease with many possible causes, clinical trials have so far focused only on the β-amyloid causative hypothesis. Since the disease progresses slowly and becomes exponentially difficult to treat in its later stages, the identification of early-onset biomarkers is critical for disease modification. GWAS studies have identified multiple gene mutations resulting in symptoms congruent with AD. However, the interpretation of these studies still relies on distilling the rate of formation and accumulation of β-amyloid and/or tau NFTs as being early-onset hallmark biomarkers, which may compound the β-amyloid hypothesis monopolistic observational bias. CRISPR represents a promising opportunity to correct this bias as well as to edit relevant GWAS-identified mutations.

Experiments in animal models have shown that CRISPR can edit the genes of interest, and slow the progression of the disease in-vivo. CRISPR has shown promising results in murine trials for finding a cure for Huntington's disease, and more information has been gained using the Rhesus macaque model for Parkinson's disease. However, considering the mechanistic differences between animals and humans, in-vitro AD disease models using 2D cultures or 3D brain organoids derived from human iPSCs represent valuable additions to analyzing human disease mechanism and progression, as well as in observing CRISPR intervention.

Such CRISPR animal or human organoid model interventional studies - such as the selected ones presented in Table 1 - have the potential to ascertain whether or not symptom development is dependent on the necessity and/or sufficiency of the formation and accumulation of β-amyloid and/or tau proteins. Extracting such information represents a quantum leap over conventional biomarker observational studies and has the potential to rapidly pivot away from false observational biases and hypotheses. In this respect, CRISPRized knockout/knockin animal models are as – if not more – important as using CRISPR as a therapeutic modality. CRISPR enabling identification of 'true' early biomarkers can then additionally be leveraged to CRISPR - silence or express relevant gene(s), thereby demonstrating the 'force multiplier' effect of CRISPR technology.

Although clinical trials of CRISPR using ex-vivo genome editing are underway, any CRISPR-Cas9 therapy for AD will need to be delivered to the central nervous system; a non-trivial exercise. Delivery platforms exist, but

they are in their nascent stages and will need further refinement for quantitative cargo delivery. Furthermore, if inserted incorrectly, CRISPR can lead to potential off-target effects and cause undesirable short and long-term effects.

It appears that, in the short term, CRISPR will prove to be an invaluable tool to correctly identify genes and biomarkers predisposing to the development of AD. Conventional disease-modifying drugs or antibodies can then be designed that may provide significantly greater efficacy in clinical trials. In the long term, subject to drug-delivery advances and more efficient and precise genome integration, CRISPR-Cas9 may well find its way into the therapeutic armamentarium to cure AD.

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Abbreviations

AChE - Acetylcholinesterase
CRISPR- Clustered Regularly Interspaced Palindromic Repeats
NFTs - Neurofibrillary Tangles
DSBs - Double-Stranded DNA Breaks
NHEJ - Non-homologous end joining
HDR - Homology-Directed Repair
gRNA - Guide RNA
EOAD - Early-Onset Alzheimer's Disease
LOAD - Late-Onset Alzheimer's Disease
SAD - Sporadic Alzheimer's Disease
FAD - Familial Alzheimer's Disease
APP - Amyloid-β Precursor Protein
APOE - Apolipoprotein E
APP - Amyloid Precursor Protein
AAV - Adeno-Associated Virus Vectors
PSEN1- presenilin-1
PSEN2 - presenilin-2
Bace1 - β-secretase
Aβ1–42 - 42nd amino acid in amyloid β peptide chain
Mt1 - Melatonin
iPSC - Induced Pluripotent Stem Cells
SaCas9 - Staphylococcus aureus
BBB - Blood-Brain Barrier
PINK1 - PTEN-induced Putative Kinase 1
ESCs - Embryonic Stem Cells
HTT - Huntingtin Gene
LLPS - Liquid-liquid phase separation
ALP - Autophagy - lysosomal pathway
IDPs- Intrinsically disordered proteins

PAMPs - Pathogen-associated molecular patterns
BBB - Blood-brain barrier
Txnip- Thioredoxin-interacting protein
RNP - Ribonucleoprotein
AVs - Aβ-positive autophagic vacuoles
CTSD - Closed System Drug Transfer
Glia maturation factor - GMF

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