A Century of Questions: Retrospective study of the controversy and efficacy of Alzheimer's disease models

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in partial fulfillment of the requirements for completion of the Polymathic Scholars Program in the College of Natural Sciences at The University of Texas at Austin

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Acknowledgements

Thanks to my supervising professor, Dr. Jon Pierce-Shimomura, who has been a great mentor during these past three years. I am grateful to be able to work with a passionate researcher and leader in Alzheimer’s disease research.

Thanks for the guidance I have received from Luisa Scott, research associate, who has helped me develop the skills, knowledge, and experience I need to be successful as a researcher at the undergraduate level and beyond.

Thank you for the support I have received from everyone in the JPS laboratory group: Sarah Nordquist, Jesse Cohn, Dawn Guzman, Susan Rozmiarek, Layla Young, Andrés Vidal-Gadea, Sangeetha Iyer.

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Abstract

Alzheimer’s disease (AD) has been a devastating challenge to the research, medical, and public community for more than a century. In 1906, Alois Alzheimer characterized it as a neurodegenerative disease associated with progressive cognitive and memory impairment. Today, the only way to conclusively diagnosis AD is by autopsy that shows hallmark accumulation of proteins throughout the brain as 1) senile plaques from extracellular accumulation of amyloid-β peptide (Aβ) and 2) neurofibrillary tangles from aberrant post-transcriptional modification of the microtubule-associated protein tau. The ability of an extra copy of APP carried on chromosome 21 to cause early onset AD in individuals with Down syndrome who are trisomic for chromosome 21 led to the amyloid cascade hypothesis. This hypothesis states that abnormal processing of the amyloid precursor protein (APP) to Aβ is both necessary and sufficient for the neurotoxic cascade that leads to neuron death and brain atrophy in the hippocampus, temporal lobe and regions of the neocortex. Only after the amyloid cascade hypothesis was proposed in the early 1990’s had research made significant headway on deciphering the molecular mechanism of disease. Many pre-clinical studies with drugs targeting APP or Aβ have shown efficacy in vivo and in vitro, but have failed in later clinical trials. Now, many will argue that the hypothesis “is intellectually flawed“ and is impeding progress. The question to be considered is whether the pharmacological industry has wasted time and money on a faulty hypothesis or whether they have been misled by model systems that fail to appropriately mimic the disease at a level necessary for translational results. It can be argued that the greatest source of debate that seems to prevent the field from moving forward remains with the experimental method: How do we generate a model system that appropriately recapitulates a disease which we do not fully understand?
In this paper, I will address the controversy and debate surrounding the century-old study of AD by reviewing the leading hypothesis of disease, the relevancy and efficacy of current and past model systems, and the advantages and disadvantages of in vitro vs. in vivo analysis for clinical outcomes. By doing so, I will provide a retrospective timeline of AD research efforts and a concluding argument for the diversification of models that could allow investigators a) to collaborate on a national and international scale, b) reach a consensus as to whether neurodegeneration and neuron death are a necessary endpoint of disease c) identify new therapeutic targets and biomarkers shared among multiple model systems d) establish a standard neuropathological examination criteria and genotype-to-phenotype analysis for human and animal models, e) address AD-specific pathologies, nondegenerative pathologies and other proteinopathies, of this multifactorial disease in vivo.
Introduction: The origin of Alzheimer’s disease

Alzheimer’s disease is frequently underdiagnosed and yet it is recognized as the most common cause of senile dementia. Senile dementia is exactly how most people would think of dementia today, a problem with thinking and memory that gets worse with age. AD is becoming more and more prevalent in our ageing population with one in nine individuals over the age of 65 diagnosed with the disease. The prevalence of AD has contributed to its status as the sixth leading cause of death in the United States and why the number of new cases rises every year.

Many people diagnosed with AD lose years of life to state of disability and dependence on family, friends, and caregivers. The duration of the illness has become a public health burden, costing America 17.9 billion hours of unpaid care for an estimated value of $217 billion in 2014. Not only do AD patients cause an extreme physical and emotional toll on caregivers, but they are also some of the most prevalent users of public health care services. It is important to acknowledge the history of AD in order to better understand how the relationship between public awareness and research has evolved to advance drug discovery efforts and the model systems that make such discoveries possible.

Alois Alzheimer, a German psychologist and neuropathologist, presented the first case of AD. In a short publication, he described a middle-aged woman, Auguste D., with progressively deteriorating symptoms of memory loss, disorientation, depression and eventually, dementia and death. In the following four years, 13 other cases were to be recognized as a type of presbyrophrenia, a term coined by Wernicke and Kahlbaum in 1863 to describe a form of senile dementia. It was not until the German neuropsychiatrist, Emil Kraepelin, published his 1910 edition of Psychiatrie: Ein Lehrbuch fur Studierende Artze that “Alzheimer’s” disease became distinguished from senile dementia. Despite the influence of Kraepelin, a clear definition
separating AD from senile dementia continues to be disputed by some psychiatrist and neurologist, who argue that the pathologies are qualitatively the same.\textsuperscript{7}

Before the 1970s, there were few people, including researchers and physicians, who recognized the prevalence of AD. In 1974, the National Institute on Aging was established and became one of the first milestones toward developing a better understanding of the biological and physiological impact of aging. Later, this organization joined with several families in 1980 to form an independent, national, nonprofit organization called the Alzheimer’s Association. The association proposed diagnostic criteria and guidelines in 1984 that were followed until a revised version was published in 2011.\textsuperscript{8} In comparison to the 1984 criteria that relies on the judgment and analysis of the physician, the 2011 revised version evaluates the disease in three stages: 1) preclinical, 2) mild-cognitive impairment (MCI), and 3) dementia. Most importantly, diagnosis now includes a preclinical stage of AD when the patient is showing no behavioral symptoms (i.e. memory loss), but is observed to have measurable changes in biomarkers, such as A\textbeta and tau, in the cerebrospinal fluid (CSF) and blood.\textsuperscript{3} When the patient begins to show memory impairment, but does not meet the clinical criteria for probable diagnosis with AD, then this patient is considered to have MCI. As of right now, there are no tests or procedures to conclusively diagnose MCI or determine the underlying cause of MCI in specific individuals.\textsuperscript{4} In 2011, the revised criterion solved this problem, but continued work will be needed before the criteria are universally accepted into physician offices.

\textit{Current treatment does not stop, slow, or reverse the disease}

In 2014, Medicare and Medicaid were expected to spend $150 billion for the care of people with AD.\textsuperscript{3} This is projected to rise to $1.2 trillion in 2050 because of the lack of
treatment that can effectively halt, reverse, or slow the disease process. Current FDA-approved drugs either inhibit acetylcholinesterase or antagonize N-methyl-D-aspartate (NMDA) receptors, treatments that only improve symptoms in a small fraction of AD patients, but do not stop or modify disease-specific progression. Here I will assess the validity of the model systems that were used to identify current therapeutic approaches and drugs for the treatment of AD in order to provide a baseline from which research efforts have developed.

Before much was known about the pathogenesis of AD, the first thing people did was look at pre-existing drugs and see if they could also be effective in an AD paradigm. AD is a multifactorial disease that is frequently also accompanied by various neuropsychiatric and neurophysiologic disorders. Many neuropsychiatric disorders are treated with cholinomimetic drugs. These drugs act to reverse the cholinergic deficiencies related to the emotional and behavioral disturbances as well as the short-term memory deficits seen in patients. Based on the success of these drugs to treat neuropsychiatric disorders that overlap with AD symptomology, many researchers have investigated the role of acetylcholine-releasing neurons in AD. Post-mortem analysis of the cerebral cortex and hippocampus from patients with AD showed that acetylcholine was consistently reduced. This decrease in acetylcholine levels was later shown to be a consequence of AD in which cholinergic neurons were found to be among the first to die in middle age. The amnesic effects of anti-cholinergics are why the cholinomimetic therapies for AD were pursued and studied in humans and human cultured cell lines. Human neuroblastoma (SH-SY5Y) cells are derived from cancer cell lines and express neuron-specific proteins important for investigation of cytoskeletal dysfunction and Aβ toxicity. This in vitro model was a vital tool for testing tacrine and donepezil, two acetylcholinesterase inhibitors approved by the FDA in 1993 and 1996, respectively. In cell cultures, tacrine was able to reduce
both forms of Aβ (Aβ40 and Aβ42) and donepezil acted to release a significantly increased amount of nonpathogenic soluble APPα (the soluble form of APP generated by α-secretase cleavage) into the medium.13,14 These results suggest that both drugs are neuroprotective against pathogenic Aβ, but the mechanism by which they confer protection is different. Tacrine functions to inhibit secretion of Aβ, whereas donepezil acts as a potent modifier of APP trafficking. Clinical trials have shown that long-term treatment of tacrine and donepezil are cost-effective treatments that decrease the likelihood and delay the time to nursing home placement in individuals at least 50 years of age and meeting criteria for probable AD.15,16 However, a recent long-term study showed that the beneficial effects of donepezil weaken after 12 months in patients with mild to moderate AD.17 The alternative, tacrine, would rarely be prescribed because of its adverse effects that cause 43% of individuals to stop taking the medication.18

The four FDA-approved acetylcholine-esterase inhibitors tacrine, donepezil, rivastigmine, and galantamine are typically used to treat mild to moderate clinical forms of AD. Rabbit, rat, and mouse models of AD were used to confirm the cytoprotective effects of these drugs against Aβ toxicity in vivo.19,20 Based on these results, it has been suggested that Aβ deposition is partly a consequence of cholinergic inactivity and may be reversible by administration of these anticholine-esterase inhibitors.21 However, the efficacy of these drugs is limited to mild to moderate stages of the disease and does not account for the full spectrum of disease stages. A noncompetitive NMDA receptor antagonist, called memantine, was FDA approved in 2003 to treat moderate to severe stages of the disease. It was shown to improve cognitive symptoms that would otherwise lead to early institutionalization and dependence on caregivers.22 Memantine action is proposed to preferentially block the pore of NMDA receptor-associated ion channels that have been open for a long period of time. This blockade is
protective against excitotoxicity, which if not inhibited, can result in overactivation by glutamate exposure, increased calcium influx, and ultimately, neuronal injury and death.\textsuperscript{23} The neuroprotective effects of memantine have been confirmed in rat models of transient forebrain ischemia or hypoxia and progressive functional neurodegeneration induced by bilateral clamping of the carotid arteries that provide oxygenated blood to the head and neck.\textsuperscript{24,25} However, structurally and functionally similar drugs to memantine have been tested for disease-modifying effects with little success due to off-target activity and toxic side effects.\textsuperscript{23} Therefore, although Reisberg et al. was able to show that a 28-week treatment of memantine was effective to reduce clinical deterioration in patients with late stage AD, many still question whether the drug can be useful for earlier stages if it only has symptomatic effects.\textsuperscript{24} The acetylcholinesterase inhibitors and NMDA antagonists that are currently being used to treat AD have relatively small disease-modifying effects and often show adverse side effects so that patient compliance can be low. Therefore, it can be concluded that although these model systems worked to identify and validate a treatment for humans, they were not useful to find genes important for disease pathogenesis or to develop drugs with disease-modifying effects. In order to make this different in the future, it is important to learn from and improve on these models.

\textit{How to model AD in a relevant way}

It turns out that there are several different patient populations of AD. The two most common are the familial form of AD (FAD) and the late-onset, sporadic form of AD (LOSAD). FAD is the Mendelian form of AD that compromises a small subset (~5\%) in comparison to the greater proportion of people affected by a late-onset, sporadic form of AD (LOSAD).\textsuperscript{1} Despite this, these FAD-based models have been important in the identification and validation of genetic
risk alleles that are central to the process of AD. Before the age of 65, some patients with FAD show consistent mutations in APP that result in abnormal processing and accumulation of Aβ.\textsuperscript{27} It is well understood that secretase activity controls the production of Aβ.\textsuperscript{28,29,30} It has been postulated that preferential cleavage of APP within the Aβ sequence by the α-secretase enzyme is neuroprotective because it decreases the likelihood that β-secretase will cleave APP to produce the pathogenic forms of Aβ.\textsuperscript{31}

In addition to APP, genetic abnormalities in presenilin (PS1 and PS2) genes are predictive for FAD. PS1 and PS2 genes encode for six- and eight-transmembrane domain proteins and have been recognized using genetic mapping and positional cloning approaches as abnormal genes responsible for FAD. Although mutations in APP are rare, PS mutations account for 30-70% of early-onset FAD cases.\textsuperscript{27} One leading yet controversial hypothesis is that presenilins interact with the protease, γ-secretase, as a cofactor influencing the γ-secretase-mediated processing of APP that results in enhanced production of Aβ peptides.\textsuperscript{32} These results suggest that individuals with this mutation get AD because of increased production of pathogenic forms of Aβ.

What’s more, individuals with a particular apolipoprotein E (ApoE) genotype were shown to have an increased risk for developing AD.\textsuperscript{33} The ApoE gene encodes for a plasma protein with a well-known role in lipid transport and metabolism, nerve regeneration and sprouting, and cell growth and differentiation.\textsuperscript{34} In 1993, the ApoE gene on chromosome 19 was discovered to be associated with increased risk for late-onset familial AD.\textsuperscript{35} ApoE has three common alleles (ε2, ε3, and ε4) in which the ε4 allele was identified as a therapeutic target based on high avidity binding \textit{in vitro} to the amyloid- β protein.\textsuperscript{35} Numerous studies have also demonstrated a dose-dependent link between ApoE type 4 (ε4) and late-onset familial and
sporadic AD across ethnic groups and populations\textsuperscript{33,36,37,38}. Over 65% of AD patients carry one or more \(\varepsilon4\) allele. There is evidence that ApoE reduces the age of onset of the disease by inducing conversion of soluble A\(\beta\) to the insoluble, fibrillar form that accumulates between nerve cells and cause neurodegeneration and death\textsuperscript{39}. These results would suggest that ApoE- \(\varepsilon4\) is an important genetic risk factor for AD and may play an isoform-specific role in the generation of neuritic plaques from A\(\beta\) peptides.

In the mid-1980’s, the microtubule-associated protein tau was recognized as the main component of neurofibrillary tangles (NFTs) and paired helical filaments (PHFs) that progressively accumulate in neurons of individuals with AD\textsuperscript{40,41}. NFTs are masses of hyperphosphorylated tau arranged in PHFs that accumulate in the perinuclear cytoplasm (just around the nucleus) of neurons as large, nonmembrane-bound aggregates. Like other microtubule-associated proteins (MAPs), tau (\(\tau\)) is a highly soluble protein known to co-assemble with tubulin and promote polymerization. It is capable of self-association into filamentous deposits that distinguish a class of neurodegenerative diseases, like AD, called tauopathies. Preliminary work has shown that rare mutations in the tau protein contribute to FAD and LOSAD susceptibility\textsuperscript{42,43,44}.

FAD-based models have been invaluable for studying the genetic basis of AD in a subset of patients. However, for the majority (~95%) of AD cases with LOSAD, patients are typically above the age of 65, show increased risk for AD with age, and typically, do not share the same genetic abnormalities as FAD patients. The non-Mendelian pattern of LOSAD is thought to be a result of multiple genetic and environmental risk factors that interact to produce a diverse phenotype. By far, the biggest non-genetic risk factor is aging. Therefore, it can be argued that A\(\beta\) is not the cause, but rather a downstream contributor\textsuperscript{2}. It has been shown that difficulty
remembering newly learned events, an inevitable part of aging, is one of the earliest symptoms of AD and this is most likely due to the progressive loss of neural connections in the area of the brain important for forming new memories. AD is fatal because of this progressive neurodegeneration that ultimately leads to brain cell death. In order to save the estimated 5.3 million Americans with AD today, research needs to continue to look at what can be learned from human studies and to use this information to guide future drug discovery efforts in humans.

**Human Studies**

Before the introduction of transgenic and gene targeting techniques, most of what was known about AD was based on the genetic study of multiple family kindreds with autosomal dominant FAD and examination of these single large pedigrees. This approach relies on the specificity and validity of diagnostic criteria, the accuracy of linkage-based methodology to identify polymorphic DNA markers, and the applicability of the positive logarithm of the odds (LOD) scores that are used to analyze the degree of linkage between a marker and a disease-associated gene. In the late 1980’s through mid-1990’s, this approach led to the identification of three causative genes for FAD located on human chromosomes 1, 14 and 21 and one genetic risk factor for LOSAD located on human chromosomes 19.

These discoveries were made possible through genetic linkage techniques, which require genotyping of several hundred non-functional genetic markers located throughout the chromosome and determination of those markers that have been transmitted from parent to offspring. Typically, DNA sequences that vary in size or sequence called polymorphisms are used as genetic markers if they show linkage to a diseased gene. The amount of recombination
between the marker and the gene of interest is then used to select markers that show little or no recombination, a good indicator that the genes are close together or linked. Selected markers can provide information about the parental origin of the gene-of-interest as well as information about the chromosomal region where the diseased gene may lie. If this marker flanks the diseased gene then it is possible to isolate a candidate region of 1 to 5 million basepairs in length. However, this is not normally the case and narrowing down the exact location of the gene of interest is a difficult as well as a tedious and laborious process.

Genetic linkage studies are best used when families with multiple individuals affected by AD inherit the disease in an autosomal dominant-manner. The clear autosomal dominant pattern of inheritance suggests that there may be a single mutant gene, instead of multiple genes, responsible for disease pathogenesis. However, there are several challenges to studying these family histories since they often have one or all cases of AD. These cases of AD can be caused by either non-allelic (noninherited), genetic heterogeneity (more than one causative gene), or etiological heterogeneity (genetic and environmental heterogeneity) factors. An example of this is seen in the Volga German kindred, a group of seven-related families with a mean and range age of onset at 58.7 and from 40 to 82 years, respectively. When analyzing this family the authors had to consider that LOSAD or noninherited AD is common, especially in late onset cases. They hypothesized that at least some of the individuals in this family have LOSAD and that these individuals with LOSAD, as well as those with non-AD dementia, can phenocopy AD. An individual that phenocopies AD is one who shows all the signs and symptoms of AD, but does not actually have the disorder and instead may have a different, but similar AD-like dementia. Ideally, linkage analysis is performed based on the following assumptions: 1) FAD is inherited in an autosomal-dominant pattern so that each child of an affected parent has 50%
chance of inheriting the mutation; 2) complete penetrance is dependent on age; 3) there are no individuals with LOSAD so that the sporadic rate is zero. Levy-Lahad et al. corrected for the variance in the Volga German kindred using a more conservative approach. This approach was based on a low (1%) penetrance model that excludes at-risk individuals so that all genotypes at the disease locus would be known. Only under these conditions were the authors able to compute significant LOD scores for D1S479, a chromosome 1 marker, with a maximum peak LOD score ($Z_{\text{max}}$) of 4.40. This means that the likelihood of a linkage occurring at this distance (from marker to gene of interest) is $10^{4.40}$ or ~25000 greater than no linkage. Significant LOD scores at this marker were also computed for five of the seven families, suggesting that there is a common disease haplotype on chromosome 1. These results were reported despite several confounding variables that could not be accounted for including missing data from deceased individuals that connected affected individuals and differences in marker allele frequencies between individuals. Although there are methods to overcome these drawbacks, many of these methods result in loss of robustness and power of maximum LOD score statistics.

Nevertheless, genetic linkage techniques were successfully used to identify important disease-associated genes as well as rare mutations in the APP, PS1, and PS2 genes. Missense mutations in the APP gene, a candidate gene for the chromosome 21 FAD locus, were found in FAD kindreds that showed linkage to chromosome 21. These results suggest that a mutation in the APP gene can cause AD. Similarly, Levy-Lahad et al. tested for known candidate genes that map to the chromosome 1 region, previously identified by genetic analysis of the Volga German kindred. The authors found a point mutation in the STM2 gene, which shows homology with 80.5% sequence identity to the human S182 gene. In 1995, the Alzheimer’s Disease Collaborative Group designated the S182 gene as PS1 and the STM2 gene
as PS2. The PS2 gene was recognized as the causative gene for the chromosome-1 linked locus for AD.\textsuperscript{46} More importantly, it was the PS1 gene that would later be shown to account for the majority of FAD cases. Sherrington et al. showed that missense mutations in conserved regions of the S182/PS1 gene cause the AD3 subtype of early-onset AD, which is recognized as one of the most aggressive forms of AD.\textsuperscript{50} These AD-causing mutations in PS1 were shown to alter the Aβ\textsubscript{40}:Aβ\textsubscript{42} ratio and processing of Notch, providing convincing evidence that PS1 encodes for the catalytic subunit of the γ-secretase enzyme.\textsuperscript{51} This central role of PS1 in processing of APP and Notch, a highly conserved receptor protein important for cell fate specification in many metazoans, is why mutations in this gene lead to adverse side effects. Together, these results have led the development of new diagnostic criteria that integrates such genetic abnormalities with biomarkers in order to characterize the full spectrum of disease subtypes and disease stages.

The diagnosis of affected pedigree members in these genetic linkage studies was achieved according to the criteria developed in 1984 by a work group for the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer’s Disease and Related Disorders Association (ADRDA). The 1984 criteria were developed to establish universal guidelines for the neuropathological assessment and clinical diagnosis of AD so to provide a uniform system that would make comparisons between human studies meaningful. It is important in clinical and genetic studies that at-risk individuals and individuals with non-AD dementias are not misdiagnosed as AD cases. However, this may not have always been accomplished in genetic linkage studies since the accuracy of the majority of these criteria depend on the conclusions of specialist in neurology or psychiatry. These individuals provide their professional opinion, which can be subjective, objective, and/or susceptible to human-error. In addition, the clinical examinations and neuropsychological tests were only applicable to living
individuals experiencing severe cognitive and memory impairments. Any results reported from these individuals could not be applied to asymptomatic, preclinical individuals. The most apparent shortcoming of the 1984 criteria was that the clinical diagnosis was limited to a “probability” of disease and could not be confirmed without postmortem analysis. Even then, post-mortem histopathological confirmation of deceased individuals was not always possible and instead, diagnosis relied on medical and family records. At the time of these genetic linkage studies, there was no AD-specific laboratory diagnostic test. Laboratory tests included electrophysiological methods (i.e. evoked potentials and electroencephalography), computerized tomography (CT), regional cerebral blood flow (rCBF), positron emission tomography (PET), magnetic resonance imaging (MRI) and examination of body fluids and nonneural tissues. All of these tests were used less to diagnose AD than to exclude other diseases.

Many patients with MCI or preclinical AD fail to meet the 1984 criteria and the more recent yet still inadequate DSM-IV-TR criteria developed in 2000. Since the discovery of the genetic risk factors PS1, PS2, APP, hyperphosphorylated tau and ApoE-ε4 in FAD kindreds, diagnostic modalities have expanded to include more distinct and reliable markers of AD in human and animal models. Biomarkers are physiological, biochemical, or anatomical parameters in fluid or imaging that can be measured in vivo to provide information about the disease-specific pathophysiological processes taking place in a model organism. It was from the more ethically challenged postmortem analysis that researchers were able to discover unique states of AD-affected brains which now guide existing and future diagnostic and outcome measures. Beginning in the late 1970s, when researchers began freezing brain samples instead of fixing them with chemicals, it became evident that people who had AD showed decreased levels of choline acetyltransferase (ChAT). These results led to the development of
acetylcholinesterase inhibitor drugs discussed earlier. Today, it is well understood that there is a limit to what can be concluded about the health and mental ability of an individual while they are alive based on postmortem analysis. In other words, it is impossible to know whether pathological processes evident postmortem were also symptomatic during life. What’s more, longitudinal postmortem studies that begin from the time of diagnosis to 20-30 years later at the time of death are time extensive and difficult to plan since less and less people are willing to donate their brain to science. Finally, postmortem studies cannot extrapolate information about earlier mild cognitive changes occurring before clinical presentation. This earlier stage is important for identifying presymptomatic or mildly impaired individuals that, like cancer patients, would benefit the most from treatment at an early stage before the presentation of significant cognitive and memory deficits. It is clear that biomarkers, epidemiological, and neuropsychological evidence with greater sensitivity and specificity are needed to diagnose the complete AD continuum and differentiate between AD dementia from other dementias.

In 2007, the International Work Group and US National Institute on Aging-Alzheimer’s Association did just that. They shifted the focus of diagnostic criteria toward biomarkers in order to emphasize “clinicobiological entity” rather than clinicopathology. The goal was to identify biomarkers that can be used to define when presymptomatic changes in the brain begin and to improve diagnostic accuracy at earlier stages for use in preventative medicine. The novelty of these criteria is the flexibility in diagnosis so that clinical dementia was no longer a requirement. Instead, biomarkers from imaging or biospecimen measurements can be used to provide evidence for diagnosis. For example, reproducible volumetric magnetic resonance imaging (MRI) is capable of taking adjoining thin slice acquisitions through anatomic regions of interest. These volumetric measurements have been used to show that the hippocampal
formation atrophies in parallel to cognitive impairment. Today, hippocampal atrophy is commonly used as a diagnostic biomarker of AD to distinguish between AD and non-AD dementias. Instead of using ionizing radiation as do x-rays, MRI uses powerful magnetic field and radio frequency pulses to provide detailed examination of the body, including organs, soft tissue, bone and almost all other internal structures. Although this imaging shows overlap in normal aging and AD-dementia, it is a noninvasive technique that can recognize the biomarker of neurodegeneration atrophy caused by loss of synapses and neurons in AD. MRI also provides better predictive value in late disease progression than other diagnostic biomarkers.

Andreasen et al. evaluated the combined potential of MRI to read cerebrospinal fluid (CSF) levels of tau, hyperphosphorylated tau, and Aβ42 as diagnostic biomarkers for AD in a large patient population of various dementias diagnosed according to the DSM-IV criteria. Together, the CSF-tau and -Aβ levels provided 90% predictive value with sufficient information to differentiate AD from non-AD dementias in most but not all subjects. Abnormal CSF- Aβ42 levels in individuals with lewy body dementia and vascular dementia reduced specificity to 67% and 48% respectively. Together, these results raised the question of: Are we really measuring what we intend to?

Initially, researchers believed that it was insoluble, fibrillar Aβ that was necessary to cause cell toxicity and neurodegeneration. In 1999, McLean et al. found that soluble Aβ correlated to the density of tau-reactive neurotic plaques and the amount of Aβ-immunoreactive plaques, both measures of the severity of disease, better than insoluble Aβ. This is not to say that fibrillar Aβ does not affect neuron health. However, it becomes difficult to ascertain whether it is soluble or insoluble Aβ that is the primary neurotoxic agent when the plaque deposits, composed mostly of fibrillar Aβ, are also closely surrounded by small, soluble oligomers. The difficulty with studying this aggregation process in detail is that the amyloid
protein is still not well understood. In very broad terms, Aβ is defined by its affinity for Congo red and thioflavin, two nonspecific dyes used for histology staining and to identify amyloid structure under fluorescence microscope. There is no uniform method to quantitate Aβ fibrils or oligomers and there has yet to be an established set of conditions for measurement. Schmittschmitt and Scholtz showed that amyloid formation, conformational stability, and solubility are pH dependent. This means that the conditions in which an experiment is conducted may adversely affect the propensity of the amyloid protein to form fibrils. As a result, studies that went about testing the same hypothesis, but did so in a different way and reported different conclusions, cannot be easily compared or used to reject a competing hypothesis.

Another imaging technique uses PET scans with radiotracer Pittsburgh Compound-B (PiB) to selectively measure insoluble, fibrillar Aβ independent of neurofibrillary tangles, soluble Aβ, or diffuse plaques. If considering the amyloid cascade hypothesis, one might predict that soluble Aβ will decrease as insoluble Aβ increases to form senile plaques. As predicted, Ikonomovic et al. showed that high affinity (3)H-PiB binding correlated with age-dependent, progressive increases in brain Aβ load of diseased individuals. However, this relationship is not always observed, suggesting that PiB may not detect all forms of Aβ in humans. A study of cognitively normal elderly subjects found that 42% of amyloid PET positive subjects had evidence of hypometabolism, a negative readout of synaptic function and density, when measured prior to amyloid positivity by \([^{18}\text{F}]\text{fluoro-2-deoxy-D-glucose PET (FDG PET)}\). The FDG-PET imaging procedure involves injection of a radioactive tracer, FDG, prior to the PET scan to enhance the differences between healthy and diseased tissue as viewed in a 3-D color image. The CHA argues that hypometabolism is common in many neurodegenerative
diseases and is a poor biomarker for differentiation of AD dementia from non-AD dementia or normal aging. Although this is true to some extent, FDG-PET is capable of identifying AD with 90% sensitivity on average.\textsuperscript{63} Postmortem analysis would be required to achieve a diagnosis of AD with 100% specificity and sensitivity. Since it is clearly unethical to sacrifice humans at different stages of the disease, the focus of researchers and clinical scientists has instead been on the development of new, safe, and effective tools and biomarkers to track relevant pathophysiological processes pre-mortem.

The biomarkers used in a clinical study are often selected based on the professional opinion of research sponsors and clinical investigators. This decision is also made with the cooperation of patients, physicians, payers and regulators. A few notable challenges for clinical research are to bring together these different ‘stakeholders’ and to create a clear and concise plan that will answer the research question and account for issues that might occur along the way. For example, an AD trial investigating the efficacy of a drug to treat late disease would require a large sample size of patients diagnosed with severe dementia. Most people at this stage would require constant care by a family member or friend, who would also need to give their informed consent along with the patient. The subject and their caretaker would have to commit to constant contact and visits for several months to years, which may not be realistic for those living far away. Inevitably, patients sometimes die and complications, financial or personal, may arise during this time period and cause the patient to withdraw from the trial. All of these events can result in a small sample size that may not be representative of the patient population at large and as a consequence adverse side effects may be overlooked. Therefore, it is important to realize that most clinical trials will not have their ideal patient population. The real difficulty then is finding a balance between what is ideal and what is feasible.
Sometimes finding this balance requires modifying or narrowing down a research question. As an alternative, researchers can use animals instead of humans and engineer them to model a human disease in a relevant and informative way. Although humans do not look and act like most animals, they share many anatomical and physiological features with them. The similarities shared between humans and animals far outweigh the differences. Based on this and what we have learned from humans, most animal models of AD are generated to carry one or more FAD-causing genetic mutations. The most common of these models is the APP overexpression model based on the amyloid cascade hypothesis. For the past 30 years, this hypothesis has been the driving feature for both formulating and learning from animal models of AD. It continues to dominate AD research despite having led drug discovery efforts in a hole these past ten years.\textsuperscript{64,65}

\textit{The Amyloid Cascade Hypothesis}

Many blame the lack of effective treatment on the closed doors of the “Church of Holy Amyloid” (CHA), an idea which drives researchers to pursue the amyloid cascade hypothesis and an amyloidocentric approach without considering other equally up-and-coming alternative hypotheses to AD.\textsuperscript{64,65} It is believed that for a drug to have disease-modifying effects, it must inhibit the pathogenesis of AD that leads to clinical symptoms including Aβ deposits, neurofibrillar tangles, inflammation, iron deregulation, and oxidative damage.\textsuperscript{66} The difficulty is deciphering whether a target may be the underlying cause of the disease, a direct or indirect downstream target, or simply a bystander, part of the normal process of ageing.

The amyloid cascade hypothesis was originally proposed in the early 1990’s and has been the leading hypothesis driving most research and drug discovery efforts for the past 30 years.\textsuperscript{67,68}
The idea that Aβ is the cause of disease has led to many drug discovery efforts to reduce or inhibit aggregation of Aβ. The two major forms of amyloid fibrils, Aβ42 and Aβ40, can be found in the CSF and blood at nanomolar levels with Aβ42 comprising the majority of Aβ in amyloid plaques from FAD patients. Although the role of amyloid in AD proposed by this hypothesis has been aggressively contested in the past, those who argue against it often fail to provide an alternative hypothesis that fills the gaps in AD etiology and pathogenesis. Ghosal et al. was the only group to propose an alternative hypothesis that states that APP intracellular domain, produced from proteolytic cleavage of APP by presenilin, is capable of initiating a series of events that lead to AD-related neurodegeneration and neuron death independent of Aβ.

Despite ongoing discussion about alternative hypotheses, no one can dispute against the fact that the amyloid hypothesis has helped to focus and structure research efforts. Transgenic models with mutations in the APP or PS1 genes are 100% penetrant to cause AD and display hallmark pathologies that are necessary to understanding the mechanism and screening for compounds that treat such neuropathological lesions. The main arguments rest on the notion that these genetic determinants only account for a small number of cases in the ever-increasing occurrence of AD and without a well-known or recognized relationship and/or pathway between the ApoE, presenilin, or tau molecules and Aβ deposition, there is a vague search for drugs. The concern is that this view of β-amyloid as the center and not a marker of AD is incorrect and misleading. Evidence supporting this argument has shown that increasing Aβ deposition does not correlate with the degree of dementia. However, if considering the idea that “correlation does not mean causation,” these results are not surprising since an initiator gene would not be expected to strongly translate to progressive cell loss and death which ultimately causes the cognitive and memory deficits of the disease. Rather, Aβ deposition is expected to correlate with
processes it directly regulates such as tangle formation. This has been shown numerous times in postmortem cross-sectional studies.\textsuperscript{56,72} Yet, critics frequently use the failed clinical trials with “amyloid” drugs as evidence for why Aβ cannot be the only initiator gene. The fact that the form of AD caused by APP gain-of-function is indistinguishable from LOSAD, suggests Aβ is one initiator gene and maybe an essential gene for LOSAD. Besides, many of the clinical trials that failed did so because of unexpected complications. This means that the hypothesis was never truly tested in these trials and any broad claims about its inaccuracy are irrelevant. Instead of completely undermining the hypothesis, researchers should continue basic research to understand the endogenous function of APP in order to identify novel targets in this amyloid initiated neurodegenerative cascade.

\textit{Making the Connection to Humans: Mouse models are invaluable}

The validity of any model is closely dependent on the ability of that model to faithfully and completely mimic the disease pathology and to provide molecular insight into the mechanism of disease. Fortunately, the APP gene is highly conserved across mammalian species. The mouse, given their genetic homology to humans, is an excellent model of amyloidopathy and is the primary model system used for drug discovery efforts. However, many drugs that claimed to show efficacy in mouse models have proven to be disappointing in later clinical trials.

Initial interest in the mouse as a genetic model of AD began in 1988 after it was recognized that Down syndrome (DS) and AD share a number of characteristic features.\textsuperscript{73} DS is a well-studied genetic disorder resulting from nondisjunction of human chromosome 21 (HSA 21) during meiosis in which homologous chromosomes fail to properly separate. Humans are
diploid organisms with each somatic (body) cell containing two copies of autosomal chromosomes (not sex chromosomes) or one set of chromosomes (haploid) from each parent. However, individuals with DS have three copies of HSA 21, which means that they also have an extra copy of genes located on HSA 21, including the human APP gene. As a result, these individuals not only have subnormal mental states, but they also display AD clinicopathology thirty to forty years earlier than non-DS individuals. Overexpression of APP due to trisomy suggests that the underlying cause of this early-onset pattern of inheritance is a gene-dosage effect, specifically of the APP gene.

The genetic link that was established between DS and AD suggested that overexpression of APP is one way to model AD. Sequence homology between Aβ amyloid peptide isolated from meningeal blood vessels of AD patients and age-matched adults with DS provided concrete evidence for the hypothesis that transgenic models which genetically mimic DS are predictable models for AD. Based on comparative mapping techniques, which look at the gene order of homologue genes, the mouse chromosome 16 (MMU 16) was determined to be orthologous to the critical region of HSA 21. The problem with using MMU16 to model HSA21 is that, unlike humans, it seems difficult to achieve spontaneous occurrence of aneuploidy of this chromosome in mice. Aneuploidy is a condition in which there is a missing or extra number of chromosomes in the nucleus of a cell. Bond and Chandley show that 2% of early mouse embryos are aneuploidy. A mating scheme was developed in the mid-1970s to address the need for reliable generation of hypo- and hyperhaploid gametes. Specific trisomies, such as MMU 16, led to the development of the trisomy 16 mouse (Ts16) with three copies of chromosome 16. The Ts16 mouse represents a model of aneuploidy that has been successfully used to study DS and FAD. The model presents with age-dependent atrophy, reduced activity of
the enzyme responsible for acetylcholine synthesis (choline acetyltransferase), and degeneration of cholinergic neurons in the basal forebrain similar to those with AD and DS.\textsuperscript{80} Although Ts16 mice display an increased level of APP transcript in fetal brain relative to littermate controls, this model did not show evidence of thioflavin S-positive Aβ deposits.\textsuperscript{73} These results could be due to the fact that MMU 16 is 1) several amino acids larger than HSA 21, 2) contains genes located on at least three different human chromosomes, and 3) lacks some genes found on HSA 21 (Reeves et al., 1986). These differences are why the Ts16 model does not fully replicate the unbalanced genetic complement that would normally be observed in individuals with DS and FAD.

Most of the other transgenic mouse models of AD are based on disease-causing mutations that underlie the genetics of FAD. The most commonly used and available models include single and multi-transgenic lines with expression of \textit{hAPP}, Aβ, \textit{PS1}, human tau or any combination of two or more. Within each of these categories, there is variation in the 1) gene or protein isoform, 2) promoter, 3) AD-association mutation, 4) insertion site, and 5) background strain used to generate the model. These differences have led to mouse models exhibiting AD-related phenotypes of varying degrees and combinations so that comparisons between studies are difficult if not impossible. Nevertheless, each model has its advantages and disadvantages, which if recognized, can be used to push forward translational research in AD.

The first transgenic mouse model with a robust AD-specific phenotype wasn’t reported until 1995.\textsuperscript{81} This transgenic model (PDAPP) was generated in C57Bl6, DBA/2J and Swiss-Webster mice strains, all of which are considered common genetic background mice ideal for safety and efficacy testing. A platelet-derived growth factor (PDGF)-β promoter was used to express human APP that included the valine to phenylalnine change at position 717 (V717P).
This point mutation had been found to cause AD in individuals of various ethnic backgrounds.\textsuperscript{47,82} This model successfully expressed human APP at high enough levels to achieve progressive, region-specific Aβ deposition, dystrophic neuritic components, gliosis and loss of synaptic density in the hippocampus and cortex.\textsuperscript{83} However, like most APP transgenic mouse models, the hallmark NFTs and PHFs did not develop.\textsuperscript{82} Nevertheless, cytoskeletal abnormalities were measured and when tested in associative and operant learning tasks, significant memory impairment was also measured to distinguish 18-21 month old non-transgenic littermate control animals from PDAPP mice.\textsuperscript{84} Similar cognitive deficits were also observed in young (6-9 month old) PDAPP mice without plaques, suggesting that the cognitive deficits seen in the PDAPP model are not age-dependent and Aβ deposition may not be the cause of disease.

The PDAPP model has been useful for testing the therapeutic efficacy of passive immunotherapy for AD and understanding the mechanism by which these antibodies confer a therapeutic benefit. Led by the amyloid cascade hypothesis, this strategy utilizes the power of the immune system to treat or reverse AD pathology has been extensively tested and shown to reduce senile plaque load in transgenic mice.\textsuperscript{85} Passive immunotherapy is a safer approach to active immunotherapy because instead of directly injecting a ‘cocktail’ of Aβ and inactivated mycobacteria, this approach uses a controlled amount of antibodies targeted to Aβ epitopes to specifically stimulate the animal’s immune system and generate anti-Aβ antibodies. The immunotherapy approach is based on the inflammatory changes that occur during AD pathogenesis. One important change to focus on shows microglia clustering around senile plaques in the brain and cerebral amyloid angiopathy (CAA) in the arterioles of living PDAPP models.\textsuperscript{86,87} Koenigsknecht-Talboo et al. engineered double transgenic mice with overexpression
of the Indiana (v71F) APP mutant form and fluorescent microglia in order to study the effects of intraperitoneally injected anti-Aβ antibody (m3D6) on microglia activation.\textsuperscript{88} The Indiana point mutation (phenylalanine for valine) was found in three generations of a family of Romanian origin who had been confirmed for FAD by autopsy.\textsuperscript{89} This APP mutation is now a common way to model AD. Moreover, this study chose to use the m3D6 anti-Aβ antibody, referred to as Bapineuzumab, which had previously been shown to bind both soluble and aggregated Aβ in PDAPP mice and is in phase II clinical trials in humans.\textsuperscript{90} They found that the rate at which microglia morphology, including the number of processes per cell and cell number, changed was partly dependent on age and antibody treatment. In Phase II human clinical trials, active immunotherapy was shown to cause meningoencephalitis in a small portion of patients.\textsuperscript{91} This adverse effect was not observed in mice prior to clinical trials, however, there is evidence in transgenic mice overexpressing mutant human APP that passive immunotherapy can increase cerebral microhemorrhages in amyloid-dense vessels.\textsuperscript{92} Nevertheless, the PDAPP model has been important for understanding the mechanism of action by which antibodies alter microglia cells and how this effect may cause meningoencephalitis in humans.

Not as common but still invaluable are transgenic mice directly expressing human Aβ peptide. These models offer a unique opportunity to study the action of Aβ independent of other APP fragments that might also contribute to AD pathology. Endogenous mouse Aβ differs from human Aβ so much so that the aggregation properties of Aβ and cleavage products (Aβ\textsubscript{11-x} rather than Aβ\textsubscript{1-42}) are different.\textsuperscript{93} Processing of mouse APP results in Aβ forms that differ from human Aβ in terms of amino acid sequence and amino-terminal endpoints that seem to alter the propensity of Aβ to form aggregates.\textsuperscript{94} These results suggest that human Aβ, not mouse Aβ, is necessary for the development of AD-related neuropathological lesions in mice. Although these
Aβ models help overcome this particular problem because they do not require overexpression of human APP to cause AD, they have limited use elsewhere. Despite showing clear amyloid pathology, there has been no published evidence for cognitive decline in these animals.95,96

Transgenic mice that co-express FAD PS mutations (PS1-A246E or exon-9-deleted variant of PS1) and the Swedish mutant form of human APP (APP695swe) were developed to achieve cognitive deficits and AD pathology, two symptoms that are not observed in single presenilin mutants.97 Consistent with this hypothesis, the multi-transgenic model showed significant neuronal loss and Aβ plaque deposition at earlier times than most hAPP mice.98 The APP695swe mutation is a double mutation (KM670/671NL) near the β-secretase cleavage site that was found to cosegregate with FAD in two large Swedish families.48 The phenotype of this double mutant mouse was important for testing the efficacy of early intervention treatments. In PS1 single mutants, chemical inhibitors of γ-secretase have been shown to reduce Aβ peptide levels.99,100 Although this has not been tested in APP/PS1 double mutant mice, they have recently been used to demonstrate the potential pathogenicity of the longer, yet just as frequently formed Aβ43 variant.101

It is important to note that only mouse models that harbor mutations in human tau show evidence of neurofibrillary pathology. However, these models fail to cause other important AD phenotypes.93 The JNPL3 transgenic mouse expressing the most common (P301L) mutant tau protein was crossed with the hAPP Tg2576 mouse to develop a model with both Aβ and tau aggregation.96 This double transgenic model showed significant induction of tau pathology in areas with high plaque load, suggesting that Aβ can augment tau pathology.102 Holcomb et al. similarly engineered a double mouse mutant for PS1 and APP by crossing the Tg2576 mouse with a mutant PS line.103 However, he found that although the transgenic mice showed
numerous Aβ deposits by 32 weeks, they did not develop complete tau pathology with clear precipitation of NFTs at this age. The inconsistency between mouse studies can be attributed to the different routes people have taken to construct models expressing identical mutations.

Here I will compare the two most common transgenic APP mice, PDAPP and Tg2576, to illustrate the significance of these differences. Among these differences, the choice of promoter is perhaps the most important since it is the primary determinant of the spatial and temporal expression of AD lesions. As discussed, the PDGF-β promoter was used in the PDAPP mouse to drive hAPP gene. In comparison, the Tg2576 mouse utilizes the hamster prion protein (PrP) cosmid vector. The former promoter localizes primarily within neurons of the central nervous system, whereas the hamster PrP cosmid vector induces a more widespread expression in neurons, astrocytes, oligodendrocytes, and microglia.104,105 A possible consequence of this is that these models have noticeable differences in onset of AD pathology such that Tg2576 mice exhibit pathology at embryonic (E) day 12.5 before PDAPP mice (E15). In addition, these models express different AD-related mutations with a common effect of overexpressing hAPP. In other words, both transgenic lines use only one mutation, but they accomplish an AD phenotype by alternative paths. The PDAPP mouse expresses a mutation in the γ-secretase cleavage site necessary for production of all three APP isoforms (APP695, APP751, and APP770), whereas the Tg2576 mouse expresses the Swedish mutation with the APP695 isoform only. Only some of these isoforms include domains such as the Kunitz protease inhibitor (KPI) domain known to interact with other proteins in the notch, tumorigenesis, and endocytic signaling pathways.106 Given these differences, it is important to note which isoforms are being expressed in each model in order to appropriately interpret results.
Even within the same transgenic line, the studies can differ based on the background strain and experimental procedure. The background strain sets the baseline for behavioral performance, which can be variable depending on the strains susceptibility to inflammation, excitotoxicity, neurodegeneration and most importantly, hAPP or Aβ effects. These differences can account for the varying degree and timing of AD phenotypes reported in mouse models based on the same genetic mutations. However, drugs that provide protection in several mouse models with different genetic backgrounds can provide insight about the robustness of these treatments that will be necessary for translation into the genetically diverse human population.

From this, it can be concluded that the mouse model is not perfect, but still remains an invaluable tool for drug discovery efforts. Mice are simple enough for preclinical settings due to their short lifespan and large litters. However, many experiments include low numbers of mice, forget to balance gender in treatment groups, fail to exclude mice that die due to unrelated causes, produce bias by keeping siblings in the same treatment group, and often, will not be reproduced before publication. These issues invariably lead to false-positives that reflect poorly on the usefulness of the model. Good experimental design based on relevant and predictive outcomes measured without bias are necessary for successful translation of drugs to humans. When studies are carefully performed, the large mutant library of transgenic and knockout mouse models of AD can be the primary method for validation of therapeutic compounds across mutant strains and throughout the continuum of AD.

*Drosophila melanogaster model of AD*

Many of the disease-causing genes, including APP, presenilin, and tau, identified in FAD kindreds have orthologs in the *Drosophila melanogaster*, commonly referred to as the fruit fly.
Historically, the fruit fly has been used for the study of aging, cancer, and several central nervous system-related disorders because of its short lifespan, large number of progeny, well-studied anatomy and phenotypes, and available tools for genetic and pharmacological manipulation. The sequencing and decoding of most of the fruit fly genome was released in 2000 and has since, allowed for an expansion in the study of neurodegenerative disease using both forward and reverse genetic approaches.

Fruit fly shares many key biological pathways with humans because of the structurally and functionally related gene families that drive these pathways.\textsuperscript{107} This conservation between humans and fruit fly is why researchers have investigated the usefulness of fruit fly to model AD. In 1989, Rosen et al. isolated and identified a nervous system-enriched transcript that encoded for a protein with strong homology in amino acid sequence and structure to the human APP gene. However, this APP-like (APPL) protein was only expressed in the nervous system, specifically in differentiated neurons, and lacked the neurotoxic $\alpha\beta_42$ domain.\textsuperscript{108} Because A$\beta$ cannot be generated naturally in the fly, nervous system-directed expression of transgenes encoding peptide $\alpha\beta_42$ in \textit{Drosophila} have been used to produce progressive learning defects, locomotor dysfunction, and neurodegeneration similar to those in mouse AD models and AD patients.\textsuperscript{109} The ability of the APP695 isoform found in humans to rescue the loss-of-function APPL mutant phenotype in \textit{Drosophila} suggests that the function of APP is a deep homologous trait that comes from a shared ancestry millions of years ago before the divergence of arthropods and mammals.\textsuperscript{109} Another shared structural and functional feature between humans and fruit fly is the $\gamma$-secretase enzyme with homology in presenilin and Nicastrin, two components of $\gamma$-secretase necessary for enzyme proteolysis of the Notch receptor and APP.\textsuperscript{110} Yet, the fly shows no or very little $\beta$-site APP cleavage enzyme (BACE) activity suggesting that although the fly
shows strong conservation in some features, it also shows extensive divergence in others. The highly conserved human disease genes and easily quantifiable behavior induced by AD pathological lesions in fruit flies is why the fly community has continued to progress in the study of neurodegenerative diseases like AD.

Within 14-15 days, adult flies expressing Aβ peptides in nervous system tissues begin to show statistically significant behavioral and cognitive defects that are quantifiable using learning paradigms. The Aβ transgenic Drosophila strain was generated using the UAS-GAL4 system, a biochemical technique that is used for targeted gene expression. The completely annotated genome of the fruit fly has made it possible to engineer a series of GAL4 lines, which express the GAL4 gene encoding the yeast transcription activator protein, in specific tissues. The main function of GAL4 is to bind an upstream activation sequence (UAS), an enhancer that is inserted next to a gene of interest, and activate transcription of that gene. In this case, GAL4 was expressed very specifically in nervous system tissue, so that cells in this tissue produced GAL4 and the Aβ peptide gene next to the UAS was transcribed. Using the Pavlovian olfactory associative learning assay, flies were trained by exposure to two odors: one odor paired with electric shock pulses and the other odor paired without. When tested for conditioned avoidance response in a T-maze choice point (a maze shaped like the letter “T” so that the fly has a straightforward choice to go either left or right), Luo et al. showed that UAS-Aβ₄₂ flies incorrectly choose the odor paired with an electroshock more frequently than control flies. After 20 days, UAS-Aβ₄₂ flies also showed severe deficits in locomotor function. These flies showed decreased climbing ability when placed in a vial and encouraged by light tapping at the bottom of the vial to climb up the wall. Whole-mount immunohistochemical staining of fly brains were used in this study to correlate the behavioral and cognitive deficits with formation of
Aβ deposits. The authors were also able to report gene effects on length of life, an outcome measurement that would have been impossible to observe in longer generation species. However, because day-3 adult flies already show some evidence of Aβ deposits, the authors could not conclude that Aβ deposits were directly causative of behavioral defects observed in day-20 UAS-Aβ42 flies. Nevertheless, this study exemplifies how the sophisticated genetics and experimental tools in the fruit fly can provide details about the cellular and molecular basis of disease that are helpful in hypothesis testing. The transgenic Drosophila strain expressing Aβ peptide was one way to model AD and test the amyloid cascade hypothesis, showing that Aβ deposits are not necessary for neurodegeneration.

Completed sequencing of the Drosophila genome has greatly facilitated the analysis of interacting gene networks that underlie the genetic and clinical heterogeneity of diseases like AD. For one, it has helped to reveal that many human disease-associated gene orthologs found in Drosophila are present as a single copy. This information has greatly aided in the efficiency of reverse genetic screens for genes important in disease pathology because redundancy, where a related gene performs the same function and compensates for loss of the gene-of-interest, is no longer a confounding factor that will hide an expected change. As a result, it is now plausible to study complex regulatory networks in Drosophila using information about the 700 well-characterized transcription factors in the fly genome.

The fly eye has been used extensively as a target for gene expression and genetic studies in Drosophila neurodegenerative disease models, including AD. Reverse genetic screens can be performed when you know the sequence of the gene and want to know the function of the gene based on the phenotype. The opposite is true for forward genetic screens when you want to know the genetic basis of a given phenotype. The development of the adult fly eye requires
careful arrangement of approximately 800 identical unit eyes (ommatidia) into a highly structured and precise hexagonal array. Shulman et al. showed that one way to model AD in *Drosophila* is to take advantage of the fact that any slight aberrations to cell patterning and trafficking are easily observed as phenotypic variations in the normal architecture of the fly eye.\(^{112}\) The authors used the fly eye as the target tissue for directed expression of human Tau\(^{V337M}\), under the control of GMR-GAL4, to investigate downstream interactors of tau. This mutant form of tau has been implicated in other neurodegenerative diseases such as familial frontotemporal dementia, a disorder closely related to AD.\(^{113}\) The authors showed that eye-specific activation of GMR-Gal4 resulted in activation of Tau\(^{V337M}\) as well as reduced eye size and roughened eye phenotype. A reverse genetic screen for abnormal eye pigmentation or morphology, which in this case, served as a sensitive readout of tau neurotoxicity, in *Drosophila* provided an opportunity to follow up AD susceptibility genes that were first identified in human genome-wide association studies (GWAS). Of the 84 fly gene homologs tested, only 9 RNA-mediated interference (RNAi) or knockdown transgenic lines were found to modify tau toxicity.\(^{112}\) These results showed that knockdown of ‘hit’ genes normalized eye pigmentation in *Drosophila* and that these genes may be protective against tau-mediated eye degeneration. Interestingly, this reverse genetic screen identified two orthologs of human AD-susceptibility genes, CD2AP and FERMT2, which were not initially reported in the human GWAS from 2010.\(^{114,115}\) These human genes have been implicated in cellular adhesion, the process of binding a cell to other cells or the extracellular matrix in humans and *Drosophila*. Cellular adhesion mutants and full-length APP overexpression mutants have the same blistered wing phenotype in *Drosophila*, suggesting that APP and cellular adhesion molecules are important for spatial regulation of cytoskeletal elements.\(^{116,117}\) The power and speed of this *in vivo* model is
exemplified here by its ability to independently validate loci important for tau-mediated disease mechanisms. It is likely that the *Drosophila* model can be used to complement and enrich the list of candidate genes from GWAS and reveal many of the novel gene networks and cellular pathways of AD pathogenesis.

With only 100,000 neurons in the fly brain, specific populations of neurons can be easily identified and manipulated using genetic and pharmacological techniques. The role of these neurons in flight navigation, sexual behavior, grooming and feeding, learning and memory, sleep and circadian rhythms provides researchers with a toolbox of sensitive readouts for large-scale target validation and high-throughput compound screens. For example, it has been shown that *Drosophila* show stereotypical behavioral responses, similar to humans, after exposure to central nervous system-acting drugs, such as cocaine and ethanol. These behaviors can be used in a forward genetic approach to rapidly and easily screen for compounds that suppress or enhance a disease-associated phenotype. Identification of such compounds with neuroprotective influence in fruit fly models of AD is an important validation step prior to further therapeutic development in mammalian models.

Pharmaceutical drug development in mouse models has been relatively inefficient and requires an enormous amount of risk, time, and expense. In the 1980’s and 1990’s, the *Drosophila* model system emerged as the preferred economic and powerful tool to perform large-scale *in vivo* screening. Following this, *Drosophila* was commonly used to narrow down a ‘hit’ library first identified in an *in vitro* preliminary screen. The low cost of maintenance, propagation of hundreds of genetically identical offspring from a single mating event, and rapid life cycle of ~10 days at room temperature allows for cost and time effective screening. These genetic and compound screens can include up to 50-100 compounds per week with a large
High throughput analysis in *Drosophila* often involves automated scoring of easily observed and quantified phenotypes. These phenotypes can be scored according to viability at different developmental stages, fluorescent markers, overt normal development or roughness of the eye. Behavioral assays of locomotor and circadian activity, for example, can be used to screen 5,000-10,000 small molecules in 2 or 3 months. Learning and memory assays important for neurodegenerative diseases would be limited to screens of only 25-50 drugs per week, still a substantial number relative to mouse studies. Overall, this higher quality identification and acceleration of lead compound discovery has greatly facilitated the identification of new therapeutic targets in the treatment of AD.

The greatest advantage of using a living organism such as the fruit fly is that it is free moving and capable of interacting with its environment. Living organisms like yeast, on the other hand, are confined to culture conditions that do not provide a complex physiological environment. In the fruit fly, compounds that show off-target activity or organism toxicity can be quickly removed based on the ability of the organism to live or to act autonomously. However, drug delivery methods available in *Drosophila* are limited according to the organism’s developmental stage. Early in fly development, drugs can be delivered in embryos via permeabilization and in larvae through dosage in solid media or dilute solution. This means that for a drug to be successfully administered and show an affect at these early developmental stages, it must be available for oral or transdermal delivery, show metabolic stability, and have low toxicity. Therefore, hit compounds are being selected for even before they are screened for an effect on phenotype. However, differences in metabolism and transport, as well as blood-brain permeability, have resulted in drugs that are toxic to humans but not flies and vice versa.
The significantly shorter life span results in severe phenotypes at early developmental stages, often before adult stages, so that fly models of AD do not always reflect the evolution of this disease with onset in the sixth or seventh decade of life. Although the fruit fly is a complex multicellular organism with a sophisticated nervous system, the phenotypes displayed by transgenic AD flies fail to fully mimic the human brain with its complex circuitry and pathophysiology. Together, the nonconserved properties and simplified physiology are why drug discovery efforts in the *Drosophila* should be used as primary screens or secondary validation screens only.

To date, there is no published drug screening efforts for potential therapeutics in a *Drosophila* model of AD. However, the success of Chang et al. to screen through 2,000 compounds in a fly model of Fragile X syndrome (FXS) and identify 15 candidate drugs, one of which later showed efficacy in a mouse model of FXS, has supported drug discovery efforts in *Drosophila* models of other genetic diseases like AD. Therefore, although flies have traditionally been used to study development, increasing evidence has supported its use in the study of neurodegenerative diseases. Like with any animal model system, differences in physiology can produce phenotypes that are not relevant to humans or human disease pathways. An understanding of the limitations of the model are important in dissecting molecular and cellular mechanisms of disease without making far-reaching conclusions.

*Worming our way to drug discovery: Caenorhabditis elegans*

In the second half of the 20th century, most *in vivo* approaches investigating the effects of mutations on the nervous system were performed in the *Drosophila* model system because of the small size and tractable genetics of the organism. However, the flies large nervous system with
about $10^5$ neurons made it logistically impossible to specify a single gene to a particular neuron and behavior. In 1974, Brenner introduced the C. elegans model system to address this weakness. The simplified nervous system of the worm contains only 302 or 383 neurons, depending on sex, and has been exquisitely described in terms of its anatomy, lineage, and intrinsic molecular profile by means of electron microscopy, Nomarski differential interference contrast microscopy and reporter gene technology. The nematode is unique to other nonmammalian systems with six holocentric chromosomes that move apart in parallel, rather than led by the centromere, during anaphase so that it was then possible to completely sequence and provide a finished genome. This information-rich genome has allowed for the development of new, powerful genetic methods specifically in the nematode.

The C. elegans is an appealing model system because of its short lifespan, transparent body, highly conserved biological processes, and rapid genetic manipulation. The transparent body of the nematode makes it possible to use in vivo fluorescent markers to track changes in morphology of cell bodies and axonal projections in live animals over the nematode life cycle from larval stage to death by senescence. There are 118 neuron classes, each of which can be studied with single neuron resolution using reporter genes. Within a matter of about 3 weeks, researchers can quickly evaluate the effect of aging on phenotype and assess the ability of a human transgene to produce an interpretable phenotype.

One of the difficulties in assessing AD-related pathology is being able to differentiate between normal aging and disease processes. A common hypothesis states that protein aggregation due to poor protein quality control is a hallmark of many age-related neurodegenerative diseases, including AD. David et al. used the C. elegans model system to show that widespread propensity of proteins to form insoluble aggregates is a normal process of
aging.\textsuperscript{136} This inherent property can provide a basis of differentiation from non-AD pathology in which aggregate-prone proteins can be used as biomarkers to signal changes in normal vs. diseased states. The authors found that homologs of proteins identified in Aβ plaques and NFTs from AD patients were also identified as aggregate-prone proteins in \textit{C. elegans}. Based on Aβ overexpression models, one hypothesis is that these protein quality mechanisms become overwhelmed and cannot keep up with the level of APP accumulation. Interestingly, the frequency of proteins to form aggregates with age was found to remain constant with increasing protein levels. This suggests that there may be a separate aspect of aging causing this aggregation. Together, these results support an alternative hypothesis that states that varying sensitivities to environmental factors and natural developmental and aging processes determine the rate of change in mitochondrial function and determine an individual’s ability to control disease-related protein pathology. This hypothesis is commonly referred to as the mitochondrial cascade hypothesis and was initially proposed by Swerdlow and Khan in 2004.\textsuperscript{137}

The essentially completed genome totaling 100,291,840 base pairs has provided a platform to compare and interpret other genomes, including humans. Completeness has been key for the construction of thousands of mutants and transgenic models that mimic human diseases for functional genomic studies that aim to associate genes with phenotypes. The availability of such a large mutant library is unique to this relatively modern model. Leaders in AD research have already taken advantage of this model to shed light on the controversial role of APP in diseased and non-diseased individuals. APP is a protein with structural features of a type I transmembrane glycoprotein.\textsuperscript{138} Studies in cell culture, embryonic chick brain, and rat olfactory system and cerebral cortex have shown evidence for a role of APP in neurite outgrowth, synaptogenesis, and wound repair.\textsuperscript{139} However, we still do not know the precise
physiological function of APP and how this changes with age. The facile genetics of the *C. elegans* model makes it an ideal alternative to study the normal function of APP either by expression of the human APP gene or mutation of its *C. elegans* homologue, *apl-1*. This endogenous ortholog, *apl-1*, is also a single-pass transmembrane domain protein, but like the *Drosophila* APPL protein, it lacks the Aβ peptide region.\(^1\)\(^4\)\(^0\) Researchers are still in the search of an Aβ peptide equivalent.

It has been shown that 60-80% of *C. elegans* genes are shared with humans and 36% of *C. elegans* proteins match human proteins.\(^1\)\(^4\)\(^1\) However, the enormous evolutionary distance between humans and worms has functional consequences on phenotype. For instance, the lack of an adaptive immune system and a circulatory system means that *C. elegans* may not be useful to study the complete pathophysiology of disease. For AD, it is impossible to study microglia activation, a major component of AD pathology resulting from Aβ plaque formation. Only when APP is initially cleaved by β-secretase will the γ-secretase complex subsequently cleave the Aβ sequence at position 40 or 42 to release the neurotoxic Aβ\(_{40}\) and Aβ\(_{42}\) forms. The absence of an Aβ peptide region supports the finding that the *C. elegans* system, like *Drosophila*, has no BACE homolog with β-secretase activity. Instead human Aβ and tau must be transformed into cell or tissue-specific lines in order to determine the neurotoxicity of Aβ forms and tau in *C. elegans*.

Fortunately, this can be done without having to use microinjections or DNA co-injections to introduce extrachromosomal arrays that are frequently lost during cell division. Mos1-mediated single copy gene insertion (MosSCI) techniques can be used to guarantee high frequency integration of targeted transgenes by homologous recombination. The *C. elegans* has an abundance of selection markers to facilitate insertion at more than 13,000 possible Mos1
Therefore, by this method, transgenic animals can be produced in a matter of weeks. The resultant transgenic animals can be used for loss-of-function and gain-of-function studies that aim to characterize gene function based on whether the gene is necessary and/or sufficient for disease pathology. Using this approach, it has been shown that although *C. elegans* cannot produce Aβ, they clearly have an alternative detoxification pathway that may be carried out by lysosomal and autophagic vesicles in response to induced expression of Aβ42 in muscles. Therefore, while nonconserved properties of the *C. elegans* limit investigation of APP processing mechanisms, the study of Aβ clearance mechanisms can be elucidated in this model.

The *C. elegans* model system pushed forward AD research with the first discovery of presenilin in 1993 by Sundaram & Greenwald. The authors performed a forward genetic screen for dominant suppressors of the Lin-12, a Notch receptor, egg laying defective phenotype and identified four genes, which they called suppressor and/or enhance of lin-12 (*sel*). One of these genes, *sel-12*, was later found to encode for a protein with 50% sequence homology to human PS1. The authors further implicated *sel-12* in Lin-12 trafficking by showing that reduced *sel-12* activity influenced Lin-12 accumulation in the apical membrane of neurons. The benefit of using a forward genetic screen to first find candidate genes responsible for rescuing the Lin-12 egg laying defective phenotype is that a hypothesis was not necessary to guide results. Therefore, this process was unbiased and provided credibility for discovery of novel targets, other than APP and Notch, mediated by presenilins. By the alternative technique called reverse genetics, Westlund et al. knocked out one or two *C. elegans* presenilin genes, *sel-12/PS1* and/or *hop-1*, to investigate the interaction and function of these genes to facilitate Notch-pathway signaling. The authors demonstrated that double homozygous mutants for *sel-12/PS1* and *hop-1* enhanced phenotypes possibly due to the redundant function of *hop-1*. These results
suggest that like humans, *C. elegans* also have more than one presenilin gene. This remarkable functional relationship between human and *C. elegans* proteins is also demonstrated by the ability of wild-type human PS1 to rescue *sel-12* loss-of-function mutants. Together, it is reasonable to conclude that human transgenes can function in a similar manner within the *C. elegans* system.

Despite only having 959 somatic cells, the *C. elegans* nematode is a sophisticated multicellular organism that represents an enormous advantage to the pharmaceutical industry and its drug discovery efforts. It is easily maintained in the laboratory at room temperature fed *Escherichia Coli* OP50 on NGM plates and has two sexes: male and hermaphrodite. Under these conditions, self-fertilizing hermaphrodites can rapidly develop from egg to adult within 3 days, producing about 300 progeny each. In this short period of time, millions of animals can be produced to meet high throughput demands. The small size of adult worms at 1 mm in length and 80 µm in diameter means that these animals can be analyzed in microtitre plates or in a single well of a 96-well plate with hundreds of non-starved animals. This technique has led to the streamlining of a few drugs that have shown efficacy in other nonmammalian and mammalian systems.

In most cases, the disruption of *C. elegans* homologs will lead to measurable phenotypes that can be easily scored. The sensitivity of the model to RNAi treatment further accelerates the target identification and validation process that is necessary for drug discovery efforts. Using a reverse genetic approach, genome-wide RNAi screens can be easily performed by either soaking animals in a solution of double-stranded RNA or by feeding with bacteria that produce double-stranded RNA (dsRNA). Ashrafi et al. validated this technique in *C. elegans* when he affected wild-type worms with a genome-wide RNAi library and screened for genes that reduced total fat...
accumulation which was visualized with vital dye Nile red.\textsuperscript{149} The majority of genes were quickly analyzed in a 96-well plate (one gene per well) and identified based on their ability to modify the phenotype. The authors found promising candidate genes that reduced fat when inactivated in wild-type animals as well as mutant animals with defects in insulin. These results suggest that reverse genetic screens are useful for pinpointing novel targets of human diseases. Unlike forward genetic approaches, the RNAi reverse genetic approach does not require further cloning and mapping and therefore, has the potential to streamline the drug discovery process to mammalian models where the homologue would be expected to have a similar effect on disease. The ability to induce RNAi and knockdown the level of specific genes at different developmental stages of the worm greatly facilitates functional genomic studies of genes important in age-related diseases like AD.

Most pre-clinical drug screens \textit{in vitro} are poor at selecting compounds with desirable characteristics for absorption, distribution, metabolism, excretion and toxicity.\textsuperscript{133} In comparison, early screening of drugs in the context of the \textit{C. elegans} system can provide important information about the permeability, toxicity and effectiveness of the drug. For a compound to have a pharmacological affect in \textit{C. elegans}, it must be packaged for delivery by ingestion, absorption through the skin, or via exposed sensory endings. Once the animal has taken up the drug, the ability of the drug to act on desired targets and exert an influence on phenotype is easily assessed in response to wholistic endogenous processes. Therefore, the use of this \textit{in vivo} model by itself increases the chance that the drug function and safety profile will be similar in the context of higher-order mammalian systems. For example, 30 drugs with established safety profiles in humans and known neuroprotective influence \textit{in vitro} were screened in a \textit{C. elegans}
strain expressing Aβ1-42. The in vivo screen for compounds with the ability to reduce Aβ proteotoxicity in vivo narrowed down the results from 30 to 6 lead compounds.\textsuperscript{150}

To improve the rate of success at which high quality lead compounds are identified, preliminary screens should be performed directly in whole animals instead of first being screened in vitro. The C. elegans model is ideal to do this because it provides a broad range of behavioral phenotypes that have already been characterized and explained. For example, exogenous serotonin (5-HT) uptake by the worm has been known to affect behavior in egg laying.\textsuperscript{151} This 5-HT controlled behavior is a sensitive readout of 5-HT signaling and can be used to identify and characterize the function of genes and/or drugs on this neurotransmitter pathway. Other behavioral assays important for the study of AD include Aβ paralysis, chemotaxis, pharyngeal pumping, and life span assays. The large mutant library and extensive behavioral readouts are further evidence for why the C. elegans model should be used more and more to keep pace with the advancing demands of medicine. From a reductionist approach, this model provides a necessary bridge point for translation into more complex, higher order mammalian systems.

\textit{Zebrafish as a model of human disease}

The zebrafish (\textit{Danio rerio}) is recently new to the research scene. In the late 1990’s and early 2000’s, it became universally recognized as a “dream system” for large-scale genetic screens of human disease.\textsuperscript{152} Like the nonmammalian fly and nematode systems, the zebrafish embryo offers a powerful tool with tractable properties for large-scale target validation and high throughput compound screens. Unique to the zebrafish, however, are its vertebrate features’ including a vertebrate neural structure and genome with highly conserved genes and
developmental pathways. The transparent and malleable embryo can be used to describe the molecular profile of cells, which can be directly accessed and tracked at single cell resolution in a living animal. This high quality resolution can be easily achieved with cell labeling or transplantation techniques. Researchers have already taken advantage of this unique system to study early embryonic events important to the field of developmental biology. To make life easier, most of the phenotypes used in these genetic screens were modular, in which one gene accounted for one phenotype without disrupting others, so that interpretation of results was easy and reassembling independent ‘hits’ into a complete pathway was simple.\textsuperscript{153} It is still unclear whether zebrafish genetics would be useful for studying phenotypes meant to mimic human diseases with sporadic (non-Mendelian) and late onset patterns like AD.

Zebrafish and humans diverged approximately 420 million years ago, and yet still share gene order in large portions of the chromosome.\textsuperscript{154} The highly conserved noncoding regions found in zebrafish genomes suggest that zebrafish and humans share key regulatory elements necessary for the differentiation of cells and the development of a complex body plan that is conserved across all vertebrates. Unlike \textit{C. elegans} and \textit{Drosophila}, the zebrafish has all of the tissues and organs that are affected in common human diseases. As a result, this model system has the potential to answer questions about how mutations affect organ form and function as well as organism homeostasis.\textsuperscript{155} A genetic screen using ethyl-nitrosourea (ENU) in zebrafish can objectively generate 7,000 mutations in 600 genes that may not necessarily cause complete loss-of-function, but instead partial loss-of-function so that more subtle effects appropriate for multifactorial diseases or syndromes are induced.\textsuperscript{156} This is important now that most human diseases are being recognized as multifactorial diseases influenced by many genes and proteins.
as well as environmental factors. In this case, the zebrafish may be clinically relevant for drug discovery efforts based on its ability to accurately mimic disease and human tissues.

The permeability of the model makes for delivery of small molecules in a water environment easy and applicable for testing potential gene and environmental interactions. This approach is important for modeling diseases, like AD, that are etiologically heterogeneous. Also important in a preclinical setting, the model requires very simple husbandry to reproduce high yields of 100-200 embryos per week that can be quickly assayed in microtiter plates. The zebrafish embryo is easily accessible to injection and antisense morpholino oligonucleotides, mRNA or transgenes, and phenotypes can be visualized and quantified by automated analysis. Based on these examples, it is easy to understand why the external fertilization and development of the zebrafish is one of the most compelling features that make the model a preferred choice for genetic studies and compound screens.

However, two important caveats of this model are that most phenotypes are 1) embryonic and 2) follow a Mendelian pattern of inheritance. These phenotypes can be either an advantage or a disadvantage to AD research. It has been well established that most cases of AD are non-heritable, sporadic and typically independent of genotype, except when carrying the genetic risk factor ApoE-ε4. Fortunately, the orthologs of ApoE as well as APP and PS1 genes are expressed in zebrafish and their patterns of expression have been defined. Many of these orthologs are gene duplicates that split the function, rather than duplicate the function, of the ancestral teleost orthologue of the human gene. This relationship is attributed to the genome duplication event that occurred during the teleost radiation 450 million years ago. As a result, genes appa and appb are duplicates to the human APP gene and the genes mapta and maptb are the duplicates to the human MAPT gene encoding tau protein.
Even though most AD genes are highly conserved between humans and zebrafish, there have been few published AD studies in zebrafish. One AD study by Newman et al. showed that incubation of zebrafish embryos in media containing Aβ peptide cause changes in neuron patterns. Studies have been able to investigate the role of PS1 in zebrafish to a much greater extent than APP because of the ubiquitous expression in the embryo and pattern of maternal inheritance of PS1. Leimer et al. used this information to show that zebrafish PS1 (zf-PS1) could replace human PSEN1 from γ-secretase complexes and that wild-type zf-PS1, like FAD-associated PS1 mutations in humans, cause selective secretion of Aβ42. The authors found that the size of the C-terminal fragment produced from proteolytic cleavage of zf-PS1 is different in zebrafish and that this difference may account for the shift in the Aβ42-Aβ40 ratio in favor of Aβ42 production. However, a number of differences between human and zebrafish are suspected and may make interpretation of results like these difficult.

Another important contribution by zebrafish has come from a study where antisense morpholinos oligonucleotides were injected into zebrafish embryo in order to alter splicing of zf-PS1 and cause FAD mutations. The production of truncated PS1 peptides produced dominant negative effects in which the normal PS1 and PS2 activity was inhibited and the embryo did not survive. Based on these result, it can be suggested that abnormal splicing, a common cellular change seen in aged cells, may be responsible for suppressed γ-secretase activity that occurs in ageing brains or individuals with LOSAD. Another more recent approach utilized the Tol12 transposable element, which can move positions within the genome, and Gal4-UAS system to generate a transgenic zebrafish with fluorescently labeled tau. This model of tauopathy was used to screen for drugs that improve tau neuropathology. Paquet et al. identified a potent inhibitor of the highly active tau kinase, glycogen synthase kinase 3β inhibitor, that reduced the level of
pathological tau hyperphosphorylation in zebrafish embryos treated 20 hours post fertilization.\cite{161}

The zebrafish has a blood brain barrier (BBB) made of endothelial cells that functions much like the blood brain barrier of higher order mammals. For the inhibitor to have any effect at all would suggest that it also has desirable properties for BBB permeability. Using time-lapse microscopy and other noninvasive monitoring techniques in vivo, it is possible to efficiently perform chemical screens in these models for rapid and direct identification of novel targets or downstream interactors in key highly conserved pathways of AD pathogenesis.

The distinct differences between nonmammalian systems and mammalian vertebrates make the zebrafish absolutely essential for filling in the gaps left by flies and nematodes. For this reason, positional cloning in zebrafish has led to the characterization of genes critical for vertebrate-specific decisions. Once the zebrafish genome has been completed, as expected in the next few years, it can be expected that drug discovery efforts will be accelerated through the model without losing quality of research.

*Why in vivo drug screens may be better than in vitro*

As pointed out thus far, there are obvious limitations to using mammalian and nonmammalian model systems to investigate the molecular mechanism of disease and screen for potential therapeutic targets and drugs with disease-modifying effects. The steady decline in research productivity from the pharmaceutical industry has forced people to question and review in vivo approaches in drug discovery efforts. Many researchers blame the productivity decline on current target-based approaches, which typically require identifying a target protein associated with human disease, developing a library of target-specific compounds and screening for compounds with disease-modifying effects, optimizing lead compounds with medicinal
chemistry, and then finally, validating the target and compound in multiple animal model systems. Progress in developing treatments is hampered by the extensive time required for traditional animal models to age before displaying histological hallmarks of neurodegenerative diseases, like AD. For this reason, culture cell lines are used as a faster alternative.

One of the main advantages of using cultured cells is that they do not have the same technical and ethical limitations as do animal and human models. Cell research relies on the generous donations of human volunteers after surgical operations, biopsies, and post-mortem. As a consequence, these samples are most likely coming from diseased organs or old donors. Once the samples have been isolated and cultured, they can be maintained as a continuous line of cells that can be readily and directly accessed. After Augusti-Tocco and Sato figured out how to preserve or rescue differentiated traits of the original cell, there was a massive development of cell models that preserve disease conditions indefinitely. Using these cell models, entire libraries of candidate drugs can be rapidly and rigorously screened for favorable metabolic and toxicity properties in a controlled environment. These screens are simple to prepare and require small laboratory space. In addition, most in vitro drug screens follow the same basic methodologies and often, use automated outcome measures for low costs and high number of replicates. Today, there are over 4,000 animal and human cell lines from over 150 different animal species available for drug discovery research.

Two of the most common in vitro models of AD include the primary culture cell lines derived from rodents and cells derived from cancer cells such as neuroblastomas from mouse trisomy 16 brains. Rabbit dorsal root ganglion neurons have been used as a model to investigate the mechanism by which the genetic-risk factor ApoE, specifically ApoE-ε4, and lipids interact to affect neurite outgrowth. One leading hypothesis is that ApoE, a lipid transport protein, is
important for peripheral nerve regeneration by modulating axon extension and myelination. Handelmann et al. incubated cells with rabbit β-migrating very low-density lipoproteins (β-VLDL), rich in ApoE and cholesterol, and showed that neurite outgrowth and branching increased, but decreased when ApoE as a free protein was added to the culture. Together, these results suggest that ApoE might affect neurite outgrowth by facilitating receptor-mediated lipid uptake and cholesterol delivery as well as reducing neurite adhesiveness so that axon projections can be directed away from the neuronal cell body toward its target. Nathan et al. replicated the experiment except this time incubating β-VLDL with ApoE-ε4 or ApoE-ε3 isoforms. The authors found that human plasma ApoE-ε4 and ApoE-ε3 incubated with β-VLDL have opposing effects on neuronal growth, suggesting that the effect of ApoE-containing β-VLDL on neurite outgrowth is isoform specific. These results are consistent with mammalian studies implicating ApoE in a cholesterol transport mechanism necessary for proper nerve regeneration, axon growth, and targeted nerve growth. Because the authors found similar differential growth patterns demonstrated by Handelmann et al., it seems reasonable to assume that cell cultures can be useful to make comparisons across trials and between studies.

It is important to note, however, that the effects of ApoE proteins could only be observed in the presence of lipids. The cell culture model used in these studies may not represent the exact physiological parameters and molecular interactions that determine cell phenotypes. Therefore, the results may not have significant predictive value for clinical outcomes. This points out another general difficulty associated with most in vitro studies. This problem concerns the inability of cell-based assays to distinguish between certain biological processes, such as apoptosis and necrosis, two important cell-death processes that are responsible for many
neurodegenerative diseases. As a result, most cell-based assays are not very sensitive and vulnerable to false-positives so that the model is not very cost effective in the long term.

Most cell cultures also cannot appropriately mimic AD pathology and often, are not adequate to assess drug activity at the level required for human trials. In cancer cell lines, for example, there is alternate cell signaling by cancer genes that could affect outcome measures so that positive results are misleading. The intracellular signaling, sometimes necessary for penetration of the blood-brain barrier, may not be fully represented in cell cultures where only 1% of the cell density in tissue is represented in culture. These differences can be exacerbated by bad cell culture practices that decrease the integrity of the cell line. Cell conditions that reflect the appropriate animal body temperature, blood electrolyte concentration, and cell-cell interactions are rarely applied. These cells lack adequate oxygen supply and biotransformation capabilities that are necessary to select for high quality drugs and predict the drug’s safety profile. The separation from wholistic endogenous processes means that in vitro studies are limited and typically, only used for preliminary risk assessment so that large drug libraries are narrowed down before further testing in animal models.

To make the cell model more relevant to human diseases, like AD, researchers started utilizing induced pluripotent stem cells (iPSCs) by transfecting cells with genes associated with the disease. This new approach was made possible by the landmark work of Takahashi and Yamanaka, who were able to achieve a pluripotent state in somatic cells by retroviral expression of a set of four genes. The authors have gone on to show that induced pluripotent stem cells (iPSCs) can be reprogrammed to differentiate into neurons under specific culture conditions and with a specific combination of growth factors. What makes this approach so powerful is that these iPSCs neurons carry the same genetic makeup as the patients carrying
disease-specific mutations. Therefore, these iPSCs neurons represent a cell model for genetically inherited diseases.\textsuperscript{169} Fibroblasts from patients with FAD caused by presenilin or trisomic APP mutations have been successfully reprogrammed into iPSC lines.\textsuperscript{168,170} In culture, these differentiated neurons produced quantifiable disease-relevant phenotypes and demonstrated resistance to modulators and inhibitors that would be expected in presenilin and APP mutants.\textsuperscript{171} However, the late-onset and genetic as well as etiological heterogeneity of AD makes it difficult to select a specific cell type in a specific region of the brain to target. For all anyone would know, the cell type selected may be one of many players influencing the disease. As a result, application of the iPSC technology is difficult and requires continued efforts made toward developing lines from patients with sporadic and familial AD.

Although it is impossible to recapitulate interactions between cells and drugs or drug metabolites \textit{in vitro}, the simplified cell model can still be invaluable for initial toxicology screening of drugs. It is arguably more economically practical and ethically responsible than \textit{in vivo} models. The thalidomide disaster of 1962, in which a sedative drug was removed after reaching the market due to severe patient toxicity, is a constant reminder to the research and medical community about the importance of safety screens. In order to identify the safest and highest quality compounds, it is important for researchers to practice good ethics and collaborative efforts so that the quality of existing cell culture models remain authentic and reliable. Because of the economic, ethical, and time constraints of using whole animal models, cell-based assays will remain a necessary and essential first step for handling the large commercial and medical demands present today.
Conclusion: where AD research needs to go

Based on this review and the model systems discussed so far, we can conclude that there is no ideal model system, *in vitro* or *in vivo*, to study human diseases. This is not to say that model systems have not had a significant impact on medicine and research, but that these models alone and independent from each other cannot provide the translational results necessary for human therapies. I believe a more broad and collective reductionist approach to drug discovery efforts will allow for optimization of both *in vivo* and *in vitro* methods. As outlined in Figure 1 (B), I propose that we diversify the models along the translational pipeline so we can ensure that the highest quality and safest drug is consistently being selected in preparation for preclinical trials and later human trials. The goal of diversifying the models is to allow investigators a) to collaborate on a national and international scale, b) reach a consensus as to whether a ‘good’ model of AD has to show neuron death and neurodegeneration as an endpoint of disease, c) identify new therapeutic targets and biomarkers and utilize them in multiple model systems, d) develop a standardized neuropathological examination criteria and genotype-to-phenotype analysis in human and animal model systems, e) address AD-specific pathologies, nondegenerative pathologies and other proteinopathies, of this multifactorial disease *in vivo*. Diversification of model systems will provide the best opportunity to achieve these goals, produce consistent results across species and increase the potential for later clinical success in humans with AD.
Figure 1. The role of multiple model systems in drug discovery efforts. In comparison to the scheme suggested by (A) Hall & Roberson (2011), (B) I propose that we diversify our model systems using a reductionist approach in order to accelerate the drug discovery process and improve the rate at which lead compounds are selected for and developed in higher order mammalian models.

Diversity is important for promoting the kind of discussion and debate that is needed to encourage collaboration on a national and international scale. Despite the leadership from a few national and international AD foundations, there is poor coordination between the US and other nations toward the development of new, more advanced methodologies to study AD. This lack of international harmonization has made it almost impossible to compare and combine analyses because researchers are not accepting of others data and secretive of their own. By diversifying the model systems used to study AD, we can improve the likelihood that one or more laboratory groups will be working in the same model and asking a similar question. If the research community was to collaborate cooperatively, then we would be able to guide future work based on expert consensus of the best methodologies, avoid wasted work from duplicate testing and encourage borrowing of knowledge and resources between laboratory groups.

We know that not every model or model system is capable of displaying all, or even some, of the clinical and/or pathological symptoms of AD. There is debate about whether the
models that do not show certain hallmark features of AD, including neurodegeneration and neuron health, are useful for genetic and pharmacological studies of AD. Many of the results from these models are challenged if they are not replicated and/or corroborated in more established models. The use of established and new models of AD across species to confirm drug efficacy or target validation is also important to reach a consensus about the endpoint of disease. The question is whether we should exclude the models, especially those that do not represent the endpoint of the disease (neuron death and neurodegeneration), from the study of AD. Although these models cannot fully recapitulate disease pathology, they have been useful to understanding molecular pathways of AD in detail. Instead, we could use parallel measurements of multiple endpoints in one of these models to validate novel targets specific to the pathway(s) being modeled. In other words, for example, there is no single mechanism by which neuronal apoptosis occurs, and it would be beneficial to evaluate multiple redundant endpoints (i.e. cytokeratins, nucleosomal DNA, cytochrome c, Apo-1/Fas) along the apoptotic cascade. The dissection of the apoptotic pathway into components defined by different endpoints can be helpful in reassembling and creating a full story. This method requires automated analysis, like that available in omic technologies (i.e. proteomics, genomics, metabolomics), to provide information about the differential predictive power of outcome measures (i.e. endpoints). It can also lead to the diversification of biomarkers outside of those traditionally used (i.e. Aβ or tau) so that we can account for the fact that AD is a complex, multifactorial disease with both genetic and environmental factors. Biomarkers identified in well-studied in vitro and in vivo systems can be used in supplementary omic studies that are more selective, including PCR or ELISA.
The goal of using multiple endpoints (biomarkers) in a model system is to identify biological pathways and toxicity genes and use this information to guide the interpretation of omic data. In this way, we can continuously search for new targets and biomarkers and use them to develop our understanding of systems biology and toxicology. This is a circular process in which we can combine results from multiple model systems to identify and validate new therapeutic targets and biomarkers and use these new targets and biomarkers in the same (and/or closely related) models to provide more sensitive measurements and reduce variability in the assessment of drug action. An understanding of the mechanism of drug action could then lead to the identification of even more novel targets that may have been overlooked when initially assessed with the original biomarkers. The advantage to using multiple model systems in this approach is that each model is different and will have different responses to a foreign substance. These different responses can then tell you something different or new about the mechanism of drug action and the safety profile of the drug. From a reductionist approach, we can step-by-step verify the safety properties of a drug in model systems with increasing complexity, beginning with the most simple cell cultures and ending with humans. As the complexity of the model system increases, it will become more likely that adverse drug affects or off-target activity will be detected. We must therefore take advantage of all the model systems in our toolbox in order to systematically assess the toxicity and activity of certain drugs and drugs with the same scaffold. This will allow us to expand our knowledge of how small molecules with the same scaffold affect organ and organism function and how we can use this information to guide further mining of molecular diversity for related structures that may also show good safety properties.

Broad toxicological and biological data recovered from multiple model systems is important if we want to replace existing approaches based on more traditional modes of thinking.
about diagnosis and therapeutic intervention. Instead of relying on the manifestation of symptoms in later stages of the disease to diagnose AD, we need to target earlier, pre-symptomatic stages. This can be accomplished by placing a greater dependence on biomarkers to signal changes in disease state. The diversification of models is important so that we can cover the full spectrum of disease and provide a standardized neuropathological examination criteria and genotype-to-phenotype analysis specific to each system. By assessment of multiple systems with one endpoint (biomarker), we can combine these results to achieve information about the predictive value of that biomarker without having to perform parallel measurements of different biomarkers that may not represent the same information. In this way, we can compare between species how sensitive a biomarker is and therefore, how useful it is as a readout of AD pathology. In many forward and reverse genetic screens, phenotypes are used as biomarkers of disease. Information from multiple model systems can be used to weed through phenotypes that may be too ambiguous or may be positively misleading in some species but not in others. The most informative phenotypes can be used as a standard for genotype-to-phenotype analysis so that comparisons within species (i.e. PDAPP and Tg2576 mutant APP mouse models) are interpretable. It is reasonable to hypothesize that outcome measures that can inform about AD pathology within as well as across species can also inform about AD pathology in humans. These outcome measures that do show promise in humans can then be used as the standard for neuropathological examination criteria. These criteria will be necessary to confirm “AD patients” in genetic linkage studies so that there are no discrepancies between studies investigating the same candidate gene or chromosomal region. If possible, such measures could be included in models of AD as a positive control. By following the best methodologies and
practicing ethical research without bias, I believe we will begin to see consistency in our results within and across species at a level necessary for later clinical success in humans with AD.

To achieve this goal, we also need to include biomarkers for other common overlapping and related disorders of AD. This is an important step because AD is known to have synergistic effects with many other aging disorders common in the general population. It is essential that before we move drugs into the large and diverse human population, we first address AD-specific pathologies, nondegenerative pathologies and other proteinopathies, of this multifactorial disease \textit{in vivo} and \textit{in vitro}. This can be accomplished by the diversification of biomarkers to include biomarkers of these non-AD diseases. This approach can be used in multiple model systems to obtain information about the propensity of proteins in models of AD to mimic other non-AD diseases and cause false-positives. It is best to approach the study of AD from a certain hierarchical view in which the more simple models are used before higher order organisms. This approach may be considered conservative, but if a simple organism can perform the job of a more expensive, riskier higher order organism, then the approach can save the research and pharmaceutical industry a lot of time and money. Instead of trying to measure the relative contribution of models, we should treat each model as an equally valuable and necessary step along the road to drug discovery. If we can achieve these goals and objectives, I believe that the cure for AD will not be too far in the future.
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Author Biography

Gabbe Zuniga is graduating as a Dean’s Honored Graduate and a member of Phi Beta Kappa from the University of Texas at Austin with a Bachelors of Science in Honors Biology in May of 2015. Gabbe was born and raised in Chapel Hill, North Carolina, and moved with her family to Southlake, Texas in 2006.

Throughout her undergraduate career, Gabbe has focused on neuroscience related research and has worked in 3 different labs, in 3 different institutes, and in 2 different countries. As a result she has had four posters, six presentations, and three planned publications. She has been awarded a Summer Undergraduate Research fellowship, College of Natural Sciences Book award, Stockard scholarship, Unrestricted Endowed Presidential scholarship, and nomination for the Barry M. Goldwater scholarship. During the summer of 2014, she spent 13 weeks working as a research assistant in the Department of Behavioral Neurobiology at the Max Planck Institute of Ornithology in Seewiesen, Germany.

Gabbe will begin her MD/PhD work on June 1, 2015 at the University of Texas Health Science Center at San Antonio. She plans to complete her PhD in neuroscience and an MD specializing in orthopedic or neurosurgical medicine. Ultimately, she wishes to pursue a full-time, tenure track academic position where she can combine her research training, medical training, and clinical experiences toward the development of new therapeutic techniques.