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EXPERT OPINION

The value and limitations of transgenic mouse models used in drug discovery for Alzheimer's disease: an update

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Introduction: The exponential growth in the world's aged population has increased pressure on drug discovery efforts to identify innovative therapies for Alzheimer's disease (AD). The long and uncertain clinical trial path utilized to test the potential efficacy of these novel agents is challenging. For these and other reasons, there has been an explosion in the generation and availability of transgenic mouse models that mimic some, but not all aspects of AD. The largely overwhelmingly positive results obtained when testing potential clinical agents in these same animal models have failed to translate into similar positive clinical outcomes.

Areas covered: This review discusses the value and limitations associated with currently available transgenic mouse models of AD. Furthermore, the article proposes ways in which researchers can better characterize pharmacodynamic and pharmacokinetic endpoints to increase the success rate for novel therapies advancing into clinical development. Lastly, the author discusses ways in which researchers can supplement, expand and improve transgenic mouse models used in AD drug discovery.

Expert opinion: The use of transgenic mouse models that recapitulate various aspects of AD has expanded our knowledge and understanding of disease pathogenesis immensely. Further success in testing and translating novel therapies from animal models into bona fide medicines would be enhanced by i) the availability of better models that more fully recapitulate the disease spectrum, ii) defining and measuring standardized endpoints that display a pharmacodynamic range, iii) building and including translatable biomarkers and iv) including novel endpoints that would be expected to translate into clinically beneficial outcomes.

Keywords: Alzheimer's disease, immunotherapy, pharmacodynamics, pharmacokinetics, tau, transgenic mice, β-amyloid

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1. Introduction

The exponential growth in the world's aged population has increased pressure on drug discovery efforts to identify novel and innovative therapies for neurodegenerative disorders. Every day, more than 10,000 American 'baby boomers' celebrate their 65th birthday and given that age is the greatest risk factor associated with the development of Alzheimer's disease (AD), the urgency to discover and develop safe and effective therapies for this debilitating neurodegenerative disorder is even greater [1,2]. Additionally, for most of the developed countries of the world, a disproportionate shrinkage in the younger population (versus the elderly) will result in additional caregiver and societal burdens. The long and uncertain clinical trial

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Article highlights.

- Transgenic mouse models of Alzheimer's disease have provided novel insights into disease pathogenesis.
- Studies in transgenic mouse models of Alzheimer's disease have yielded novel hypotheses that have advanced into clinical development.
- A more thorough characterization of pharmacodynamic and pharmacokinetic endpoints in transgenic mouse models of Alzheimer's disease will enhance the probability of clinical success.
- Greater use of small animal imaging modalities may increase the identification of clinically translatable endpoints.
- Further improvements in transgenic mouse models of Alzheimer's disease are needed in order to overcome the limitations in transgenic models that are currently available.

This box summarizes key points contained in the article.

path utilized today to test the potential efficacy of novel agents in human subjects is challenging. Therefore, a more appropriate use of well-validated animal models that recapitulate critical aspects of disease progression has the potential to greatly enhance the probability for successful clinical outcomes.

Since the generation of the first transgenic mouse model that displayed some (but quite minimal) characteristics of AD neuropathology, there has been a virtual explosion in the generation and availability of additional models that display more aggressive phenotypes in terms of neuropathological sequelae [3-6]. The majority of these transgenic mouse models rely on the overexpression of gene constructs containing mutations that are associated with familial forms of AD (FAD) and/or 'humanization' of endogenous genes. Collectively, most of transgenic mice engineered to express human genes do recapitulate the characteristic accumulation of β-amyloid (Aβ) in a brain region-specific manner but show minimal evidence of neurodegeneration or hyperphosphorylated tau [6]. The exception to the presentation of these milder phenotypes is the robust and aggressive neuropathology observed in transgenic mice that over-express more than one gene associated with FAD [7-9]. In addition to using immunohistochemical techniques to characterize and quantify various brain pathologies, it is now a matter of routine to also measure biochemical correlates of these neuropathological features in brain homogenates or cerebrospinal fluid (CSF; **Figure 1**, [10]). Indeed many, if not all, of the current Phase III compounds in clinical development for the treatment of AD have demonstrated positive results in these same transgenic animal models [11-16]. Disappointingly, however, there have been recent Phase III failures with clinical candidates that have also demonstrated desired effects in these same models but have failed to translate into similar positive clinical outcomes [17,18]. Prosecuting new targets and novel approaches for the treatment of AD presents many challenges that include i) the protracted duration of AD with pathophysiological correlates that remain largely ill-defined, ii) the complexity associated with unknown genetic and environmental risk factors, iii) the instruments that define the clinical course of AD are largely subjective and iv) the challenge with identifying and validating relevant biomarkers that are associated with the natural history of the disease and/or related to treatment efficacy. The lack of success in translating preclinical results from animals to favorable clinical outcomes in subjects with AD should give us pause for thought and reassessment of the value, challenges and limitations that these experimental models hold.

2. Novel insights into AD mechanisms

2.1 Focusing on neuropathological endpoints

Significant progress in understanding the pathophysiological mechanisms associated with sporadic AD has been derived from studying autosomal dominant familial forms of AD that result from the inheritance of mutations found in three genes: amyloid precursor protein (APP), Presenilin-1 (PS1) and Presenilin-2 (PS2) [19,20]. Mutations within these genes alter the production of a heterogeneous pool of β-amyloid (Aβ) peptides such that longer and more profibrillogenic species are produced. Abnormal accumulation of Aβ peptides in brain regions critical for learning and memory forms the basis for the development of the 'amyloid cascade hypothesis' of AD pathogenesis [21]. This hypothesis posits that accumulation of these Aβ peptides in brain is correlative if not causative to disease progression. In aggregate, there are now well more than 100 gene mutations, the majority of which are located in PS1, which are associated with FAD [19]. Although FAD represents only rare (≤ 5% of AD is inherited) autosomal dominant forms of AD, an extension of the amyloid cascade hypothesis to include the more sporadic and common late-onset form of the disease is supported by the nearly congruent clinical and neuropathological presentation observed in both populations. Significant progress has occurred in our understanding of how APP is metabolized with the identification and characterization of the aspartyl proteases (β-APP-cleaving enzyme: BACE and γ-secretase) that are responsible for the sequential cleavage of APP into Aβ peptides [22]. The confirmation that PS1 contributes to the active site aspartyl protease within the γ-secretase complex and that mutations within the APP gene can affect the catalytic rate at which Aβ peptides are generated further rationalizes this pathway as a viable and fruitful route of investigation. Indeed several promising small-molecule brain-penetrable compounds targeting these enzymes with drug-like characteristics are entering the early stages of clinical development [23,24]. These potential clinical candidates target the direct inhibition of BACE or modulation the γ-secretase complex in an effort to block the initial step in the generation of Aβ peptides or modulate the Aβ product profile, respectively.

The first transgenic mouse model to demonstrate accumulation of extracellular Aβ expressed a non-mutant form of APP under the control of the neural-specific enolase

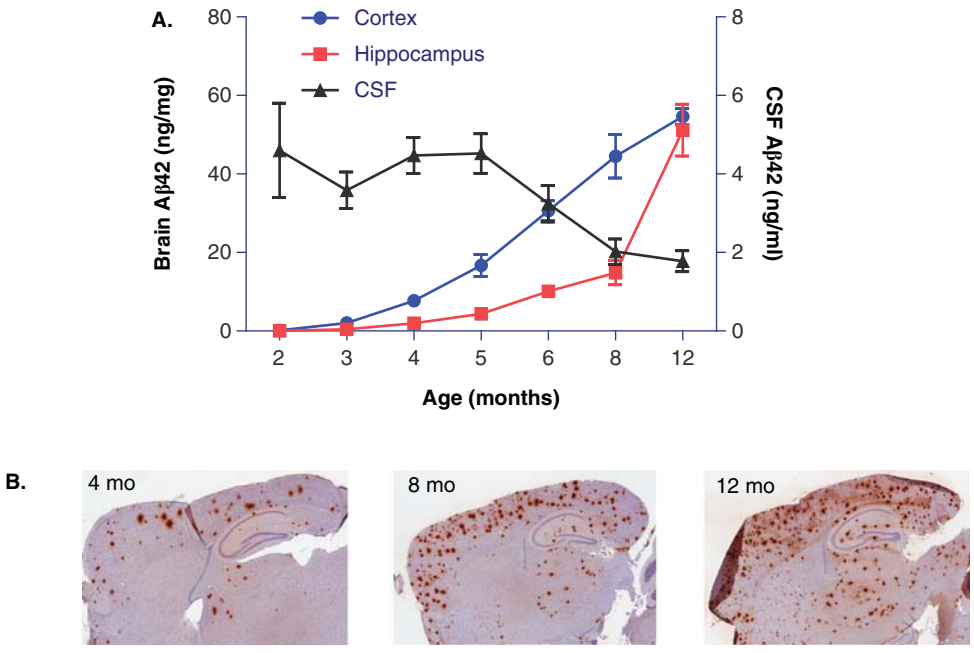

Figure 1. Age-dependent accrual of Aβ42 in hippocampal and cortical brain extracts from APP/PS1 mice as levels of CSF Aβ42 decrease (A) also correlates with immunohistochemical detection of brain Aβ (B).

promoter [3]. Shortly after this initial report several laboratories reported the derivation of transgenic mice that demonstrated robust brain region and age-dependent accrual of Aβ peptides [4,5]. Differences between the human and mouse primary amino acid sequence within the Aβ peptide region itself, the inclusion of mutations associated with FAD as well as the use of a strong promoter to drive transgene expression were key elements that probably influenced the presence of robust brain region- and age-dependent accrual of Aβ peptides.

While transgenic mice over-expressing mutations associated with FAD resulted in accrual of the Aβ peptides in brain, accumulation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau was absent. Transgenic mice expressing full-length human tau resulted in abnormal distribution of hyperphosphorylated tau in a few neurons, but similar to the initial transgenic mice expressing wild-type human APP, overt neuropathology in these mice was limited [25]. The identification of mutations within the microtubule-associated protein tau (MAPT) gene that are causative to frontotemporal dementia (FTD) without accumulation of Aβ in brain established a causative role for hyperphosphorylated tau in dementia [26]. A further delineation of how Aβ and hyperphosphorylated tau, two defining neuropathological characteristics identified in AD brain, may interact occurred when APP and tau transgenic mice were crossed resulting in the clear exacerbation of the NFT phenotype [26-28]. Furthermore, when synthetic human Aβ peptide or extracts from APP transgenic mouse brain were injected directly into the brains of tau transgenic mice, NFT pathology is exacerbated [29,30]. Conversely when Aβ levels are reduced via an immunization protocol in APP transgenic mice also expressing tau, NFT pathology is reduced [31]. Additional transgenic mouse studies support that Aβ may interact with endogenous tau to mediate neuronal dysfunction that manifests at the behavioral level [32]. Knocking out endogenous mouse tau reduced Aβ-induced cognitive impairments and increased resistance to the epileptogenic agent pentylenetetrazole in transgenic mice expressing human APP [32]. Taken together, these data suggest that accumulation of Aβ in brain plays a necessary role in promoting further downstream events involving disturbance of normal tau function and development of tau pathology.

Although tau plays an important intracellular function in stabilizing microtubules, tau can also be measured in CSF [33]. Additionally, reports have suggested that CSF tau levels may change during the course of acute or chronic brain injury [34,35]. Using *in vivo* microdialysis, interstitial fluid levels (ISF) of tau in wild-type and tau transgenic mice were significantly (~ 10-fold) higher than the levels that were measured in CSF [36]. These results are similar to those reported for ISF Aβ levels in APP mutant mice and indicate that the appearance of NFT pathology, like Aβ plaques, resulted in a decrease in the levels of the soluble ISF protein pool [37]. The ability to measure extracellular ISF tau places into context the more recent hypotheses suggesting that extracellular tau aggregates can contribute to a propagation phenomenon in select neuronal populations [28]. More intriguingly is the possibility that various amyloid protein aggregates (prion, tau, α-synuclein, Aβ) associated with neurodegenerative disorders may also 'imprint' each other contributing and leading to subsequent 'spreading' of an NFT phenotype [38,39].

2.2 Dynamics of Aβ in brain revealed by APP transgenic models

Further insights into the dynamics of Aβ efflux from the brain have been elucidated when investigating the trafficking of Aβ from brain to the periphery utilizing transgenic mice that over-express Aβ exclusively in brain [11,40]. Following passive immunization with a high-affinity anti-Aβ antibody, a shift in the equilibrium of Aβ from the brain, where it is abundantly synthesized, to the periphery was hypothesized to be the mechanism of action that resulted in the subsequent clearance of brain parenchymal Aβ [40]. A dramatic rise in plasma Aβ was observed in transgenic mice administered anti-Aβ antibody indicating the assay of plasma Aβ as a potential clinically relevant and translatable biomarker. Indeed, a similar increase in plasma Aβ was observed when a humanized version of this same anti-Aβ antibody (Solanezumab) was administered to AD subjects [41]. Moreover, increases in plasma Aβ levels have now been reported for other anti-Aβ immunotherapies lending support to the hypothesis that Aβ may be effluxed from brain following peripheral antibody administration [42]. Whether or not AD subjects will derive clinically meaningful benefit following administration of anti-Aβ antibodies is currently under investigation and the Phase III results from some studies are anticipated during the latter half of 2012 [24].

Although Aβ deposited in brain parenchyma in the form of plaques had been reported to disrupt the dynamic egress of Aβ from brain to the periphery in transgenic mice, a similar appreciation of how Aβ deposited in brain would influence the normal clearance route of Aβ in humans did not occur until recently [43,44]. Using stable isotope labeling kinetics (SILK), Bateman and colleagues [44] have now demonstrated that AD subjects show no difference in the *de novo* synthesis rates of Aβ but rather have a significant decrease in the fractional clearance rate of Aβ [44,45]. Although the pool of Aβ that is labeled using SILK is small, the overall difference in clearance rates of both Aβ 40 and Aβ42 between control and AD subjects was significant and could, over the lifetime of an individual, represent a significant sequestration of a large reserve of Aβ in brain. By probing the interstitial fluid (ISF) brain compartment of Aβ levels in young versus plaque-bearing APP transgenic mice, further insights into the dynamic equilibrium between ISF and plaque associated Aβ have been revealed [46]. Following acute administration of a γ-secretase inhibitor (GSI) to APP transgenic mice that had a substantial plaque burden, a significant decrease in the levels of the shorter Aβ40 and Aβ38 peptides was measured with less of an effect on the longer more profibrillogenic Aβ42 species [46]. By combining anti-Aβ passive immunization with suppression of Aβ formation in a conditional FAD over-expressing transgenic mouse model, a significant decrease in brain Aβ burden was observed when antibody treatment was initiated in very old plaque-bearing mice [47]. The significant reduction in brain Aβ levels occurred for all species of Aβ and also resulted in a significant reduction in the level of microgliosis [47]. Taken together, these studies suggest that there are multiple pools of extracellular Aβ peptides in brain that, depending on the level of plaque burden, can act as reservoirs for subsequent deposition. Importantly, these initial observations also suggest that in addition to blocking *de novo* production of neurotoxic Aβ peptides, it might also be critical to drive clearance of deposited or loosely associated species simultaneously in order for either therapy to be efficacious.

2.3 Dominant role for clearance of brain-derived Aβ by apolipoprotein E

An important and surprising role of apolipoprotein E (apoE) in promoting the clearance of Aβ from brain was elucidated by crossing transgenic mice that over-express APP mutations to mice that express each of the three isoforms of the human apoE gene [48]. The resulting bigenic mice expressing both human Aβ and apoE recapitulated the apoE4 isoform-dependent increase in Aβ levels in both the brain and the microvasculature [49]. However, the overall amount of Aβ that accumulated in brain parenchyma, even in old APP/apoE4 mice, was less than that when murine apoE was present [50]. Using sensitive ISF Aβ measurements, bigenic mice expressing APP and human apoE4 but not apoE3 have a prolonged half-life of extracellular Aβ [51]. In humans, both CSF and imaging biomarkers have suggested that apoE4-dependent mechanism(s) that retard efficient clearance of brain-derived Aβ may contribute to the genetic susceptibility apoE4 confers to AD [51].

2.4 Focusing on the synapse

The overwhelming majority of studies probing various mechanisms associated with AD pathogenesis quantify endpoints involved with Aβ and/or hyperphosphorylated tau accumulation [52]. While relevant to the disease process, the most robust correlations to cognitive decline in AD subjects is synapse loss [53,54]. Although many FAD APP transgenic mouse models do not show overt neuronal loss, synaptic loss has been reported in different models [55]. Interestingly, in Tg2576 transgenic mice, prior to the appearance of robust brain Aβ accrual, a significant reduction in mushroom-type spines in basilar but not apical dendrites of CA1 pyramidal neurons were observed [56]. This reduction in basilar spines was accompanied by a concomitant decrease in GABAergic interneurons suggesting that disinhibition in this region of the hippocampus may contribute to an overarching hyperexcitable state [57]. These results are quite congruent with emerging fMRI data from older, cognitively normal individuals who have evidence of brain amyloid deposition and who demonstrate a lack of connectivity in the default mode network that includes regions of the hippocampus and medial temporal lobe [58].

2.5 Neurogenesis

Changes in synaptic function and plasticity in AD may involve alterations in adult neurogenesis. Several studies using FAD APP transgenic mouse models have shown altered neurogenesis in the subgranular zone of the dentate gyrus as well as in the subventricular region of adult brain. However, results have been variable depending on the FAD mutation, transgene construct, age of animal and markers used to define neural progenitors and their subsequent proliferation [59]. Decreased neurogenesis in the hippocampus of adult APP transgenic mice has been reported in several studies whereas other studies have reported an increase in neurogenesis in transgenic mice [60-66]. Some of the variation in neurogenesis in the different animal models may also arise from differential effects on neurogenesis of APP metabolites such that Aβ may reduce survival of progenitor cells [61], whereas soluble fragments of APP such as sAPPα may promote neurogenesis. Since neurogenesis has been shown to be important for synaptic plasticity and hippocampal-dependent learning and memory [67], these findings have encouraged further investigations of neurogenic regenerative mechanisms as potential therapeutic targets for AD.

3. Novel therapeutic approaches for drug discovery revealed by animal models

3.1 Active immunization approaches revealed by APP transgenic models

Novel and unexpected insights into active immunization approaches using the Aβ peptide as an immunogen were first elucidated in the PDAPP transgenic mouse model of AD-like pathology [11]. Instead of exacerbating or promoting the deposition of Aβ in the brains of younger pre-plaque PDAPP mice, immunization with Aβ42 emulsified in complete Freund's adjuvant resulted in the complete blockade of Aβ deposition. Even when the immunization protocol was initiated in older plaque-bearing mice, there was a significant decrease in the amount of Aβ that was measured either biochemically using a sensitive ELISA or immunohistochemically. Additionally, the accompanying gliosis and neuritic-plaque phenotype characteristic of this transgenic mouse model was greatly reduced following immunization [11,68-70]. Active immunization with various fragments of the Aβ peptide in a variety of transgenic mice over-expressing Aβ have confirmed and extended this initial finding [71]. Indeed the robust and reproducible preclinical findings in APP transgenic mouse models triggered clinical trials using aggregated Aβ in a vaccination paradigm. In an initial Phase I clinical trial that enrolled 80 subjects with AD, no adverse effects were observed and subjects that mounted a titer to Aβ appeared to display a slower rate of cognitive decline when compared with non-titer responders. Despite these promising and exciting early results, a subsequent Phase IIa clinical trial (designated as AN1792) was halted prematurely due to the appearance of meningoencephalitis in a significant portion of subjects that developed titers against Aβ. Follow-up autopsy analysis of subjects in the AN1792 trial who had generated measurable titers against Aβ42 demonstrated a striking removal of Aβ from brain parenchyma as well as amelioration of neurite abnormalities and hippocampal tau pathology similar to the results documented in preclinical animal studies [72,73]. Although cognitive decline did not appear to be halted in the AN1792 trial, the robust removal of parenchymal Aβ has bolstered continued support for active vaccination approaches for the treatment/prevention of AD. Currently several active clinical trials are underway that target minimizing T-cell responses in an effort to circumvent unwanted meningoencephalitis [74].

3.2 Passive immunotherapeutic approaches for AD

A significant reduction in brain Aβ burden was also observed when anti-Aβ antibodies were administered passively to APP transgenic mice [12,75,76]. Indeed similar to the results observed following active immunization of APP transgenic mice with Aβ42, passive immunization with anti-Aβ antibodies directed toward various regions of the Aβ peptide was just as effective at preventing brain Aβ accrual [71]. Additionally, certain anti-Aβ antibodies even when administered to extremely old transgenic mice were able to reduce the brain Aβ burden as well as reverse spatial memory deficits in a water maze learning paradigm [77]. One proposed hypothetical mechanism of action of passive immunization suggests that antibody enters the central nervous system and binds to deposited Aβ in brain subsequently triggering Aβ removal by Fc-mediated activation of phagocytosing microglia [75,76]. In support of this proposal, a significant amount of anti-Aβ antibody decorating plaques has been observed [47,75]. While binding to plaques in brain may be one possible mechanism whereby passively administered anti-Aβ antibodies are able to effectively remove Aβ, other mechanisms are also possible since a similar dissolution of plaque can be observed when APP transgenic mice are administered $F(ab)_2$ fragments that lack the Fc region from some of the same anti-Aβ antibodies proposed to act via microglial mechanisms [78].

Based on these exciting and promising results observed with passive immunotherapeutic approaches in APP transgenic models, several late-stage (Phase III) clinical trials are underway. The most advanced passive immunization approaches utilize anti-Aβ antibodies that target the N-terminus (bapineuzumab) or mid-domain region (solanezumab) of the Aβ peptide. Additionally, several passive immunization approaches targeting oligomeric and/or conformational structures of the Aβ peptide are also advancing through various stages of clinical development [74]. Preliminary encouraging results in small Phase II trials with bapineuzumab or gantenerumab using carbon-11-labeled Pittsburgh Compound B (^{11}C-PIB) positron emission tomography (PET) suggest that both of these agents are affecting removal of Aβ from brain [79,80]. ^{11}C-PIB is an imaging agent that selectively labels thioflavin S amyloid deposits in the brains of living subjects and as such can serve as an accurate indicator of

potential treatment effects [81]. In both Phase II studies, the number of subjects that were treated was too small to reach any conclusion based on cognitive improvement [79,80]. Although promising, these initial results should be tempered by the observation of an increased incidence in the appearance of vasogenic edema that was most prominent in apolipoprotein E4 carrier subjects receiving the highest dose of bapineuzumab [82,83].

3.3 Potential for tau immunotherapy

Both active and passive immunization approaches appear to be effective at attenuating the biochemical, pathological and behavioral phenotypes characteristic of transgenic mice over-expressing tau mutations. Different laboratories using different immunization protocols and tau transgenic mice report reductions in tau pathology in brain as well as attenuation of behavioral deficits [84]. Passive administration of either a conformational or phospho-specific anti-tau antibody significantly attenuated the accrual of insoluble tau as well as reversed the detrimental motor phenotype in transgenic mice over-expressing mutated tau [85]. While further work is required to more carefully elucidate the mechanism(s) whereby these benefits are occurring, these preliminary data in tau transgenic mouse models suggest that active and/or passive immunization approaches targeting tau may, like Aβ immunotherapy, have potential clinical therapeutic application.

4. Limitations of current animal models

4.1 Lack of overt neurodegeneration

Despite the availability of numerous transgenic mice that recapitulate various aspects of AD neuropathology, these animal models have significant limitations. Transgenic mice over-expressing various mutant forms of APP and PS1 genes have provided facile tools with which to probe the dynamics of Aβ synthesis and clearance but the lack of overt neurodegeneration, even when extracellular levels of Aβ42 reach ~ 60 ng/mg, is problematic (**Figure 1**). More aggressive transgenic models expressing up to five different mutations associated with FAD can overcome the lack of neurodegeneration as intracellular accumulation of Aβ appears to be more toxic [9]. Recently, transgenic mice that over-express Aβ42 in the endoplasmic reticulum demonstrate overt neurodegeneration in the hippocampal CA1 region; however, identification of neurons in the AD brain with intraneuronal accumulation of Aβ is difficult [86]. Moreover, the scarcity of neurons in AD brain that are positive for intraneuronal Aβ suggests that although detrimental to normal neuronal function, intraneuronal accumulation of Aβ is unlikely to drive the profound neurodegeneration associated with AD progression [87]. NFTs composed of hyperphosphorylated tau, a defining neuropathological characteristic found in the brains of AD subjects, are also absent in transgenic mice that over-express FAD APP mutations. The clear correlation between NFTs and cognitive status in AD subjects highlights the importance of addressing this discrepancy in models that might be developed in the future.

4.2 Incomplete understanding of the role of neuroinflammation

Scientific evidence derived from studies in experimental AD preclinical models has resulted in recognition that the role of neuroinflammation in AD pathogenesis is complex, exhibiting both beneficial and detrimental consequences. Both APP and tau transgenic mice develop pathology that is accompanied by microglial and astroglial activation as well as cytokine production [88]. Glial activation has been shown to reduce plaque pathology by a mechanism involving an increased Aβ clearance [89-93]. However, microglia may also produce detrimental pro-inflammatory cytokines that impede efficient Aβ clearance, promote tau phosphorylation, neuronal injury and cognitive impairment [94-96]. In order to explore and identify potential therapeutic targets, it is clear that further studies are needed to understand the spatial and temporal order of neuroinflammatory events that occur at different stages of AD pathogenesis. In this respect, transgenic mouse models have enabled profiling of neuroinflammatory changes during the development of plaque and NFT pathology [97-99]. Such studies may aid in the identification of critical signaling pathways underlying proinflammatory versus neuroprotective states of microglia and astrocytes.

4.3 Challenges with translating behavioral phenotypes in APP transgenic models

The ability to understand and place into context the pharmacological reversal of aberrant behavioral phenotypes typically associated with transgenic mice over-expressing mutations associated with FAD is a major challenge. Indeed complete reversal of Morris water maze deficits in transgenic APP mice dosed with various anti-Aβ antibodies resulted in a robust and significant reversal of deficits such that transgenic mice treated with these anti-Aβ antibodies performed nearly as well as untreated wild-type litter mates [76]. An acute reversal in object recognition and hole board learning deficits following administration of an anti-Aβ antibody to very old PDAPP transgenic mice demonstrates a greater need to understand how the Aβ peptide induces such profound memory deficits that can be reversed rather rapidly and without an overt effect on parenchymal Aβ levels in brain [100]. Although Phase III clinical trials are still underway, to date there have been no reports of cognitive improvement and/or stabilization with any of the agents under investigation. Recently, a Phase III trial probing the efficacy of a GSI (semagacestat) was prematurely terminated due to the unexpected appearance of cognitive worsening in AD subjects given test article [24]. More concerning is the report that this cognitive decline continued after termination of the clinical trial, during the follow-up period. This same class of GSI compounds, when studied in transgenic mice demonstrated robust decreases in brain Aβ levels as well as significant reversal of

memory deficits [101]. No reports of behavioral impairment following administration of GSIs to transgenic or wild mice are available. Taken together, there is a clear need for a better understanding of the exact cause of cognitive deficits in AD subjects that can then be more appropriately modeled in preclinical animal models.

4.4 Paucity of data using imaging endpoints

Significant advances in small animal imaging modalities make it possible to incorporate these endpoints into studies using pharmacological manipulation or during initial characterization of a novel transgenic line of mice. A summary of studies using various imaging modalities and transgenic models is provided in **Table 1** and **Table 2.** [102-126]. PET has been used extensively in drug discovery efforts to gauge the level of orthosteric target engagement. As a future prospect, application of PET ligand receptor occupancy in various transgenic mouse models of AD could be informative with respect to the state of various neurotransmitter systems. Although it is assumed that ^{18}F 2-deoxy glucose (2-DG) PET studies in APP transgenic mice will be challenging, a significant hypometabolism has previously been reported to occur using 2-DG autoradiography in PDAPP transgenic mice [127]. Changes in ^{18}F 2-DG PET are well documented to occur in AD and are also correlated with apolipoprotein E4 carrier status [128]. Volumetric magnetic resonance imaging (vMRI) of AD subjects is routinely used in clinical trials and similar vMRI endpoints have been utilized to characterize the significant volume loss in both the hippocampus and cortex in the Tg4510 tau transgenic mouse model that was also correlated with the aggressive neurodegeneration observed in this model [129]. Magnetic resonance spectroscopy (MRS) utilized to probe the brain neurochemistry in this same study revealed significant alterations in the myo-inositol: total creatine ratios. These metabolic changes were proposed to be indicative of the attending gliosis observed in this tau model. Few studies with transgenic mice have incorporated functional MRI (fMRI) endpoints; however, emerging human data suggest that alterations in fMRI may represent a leading indicator of poor brain health and may even provide a facile modality to probe treatment-related efficacy in rather small cohorts of humans [130,131]. Although implementing similar fMRI paradigms in transgenic mice is challenging, the ability to probe functional connectivity of various brain networks may reveal novel insights into disease-causing mechanism(s). In aggregate, the development of small animal imaging modalities now makes it possible to incorporate these endpoints into transgenic mouse studies to characterize new and existing animal models and/or to probe pharmacological interventions.

5. Proposed improvements

5.1 New models are required

While current transgenic mouse models of AD have provided novel and important insights into disease pathogenesis, the lack of a full neuropathological phenocopy of the AD brain needs to be addressed. Given the possibility that the Aβ profile even in very old (36 months of age) transgenic mice that vastly over-express FAD mutations still represents only the phenotype of Aβ that may be present in early/mild and sporadic stages of AD pathogenesis, other features will need to be considered. In this regard, recent models that express biochemically modified forms of Aβ, that is, pyroglutamate Aβ, appear to cause a more profound and robust neurodegenerative phenotype [132,133]. Additionally, a greater appreciation of the role that other fragments of APP (like sAPPα) may play in neurogenesis must be considered when evaluating neurodegeneration in a transgenic mouse model that also overexpresses these fragments. Indeed, transgenic mice that have been engineered to express human APP and PS sequences at endogenous levels demonstrate a lower level of Aβ accumulation but show altered neuronal phenotypes [134]. Although overt neurodegeneration is significant and quantifiable at the end stage of AD, the realization that more subtle and neurotoxic effects of a heterogeneous pool of soluble Aβ peptide (s) may actually be the causative culprit is emerging [135]. In addition to enhancing our knowledge with regard to more subtle effects of human Aβ in rodent brain, consideration must also be given to generating and characterizing transgenic mouse models that express more than one gene associated with AD. Since important species differences between the human and mouse coding sequence of genes implicated in AD have been identified, a mouse expressing human apolipoprotein E4, APP as well as tau should be informative and may even result in an animal model that more fully recapitulates the constellation of neurodegenerative events that are disease relevant. While generating a 'triple' transgenic mouse with fully humanized genes expressed at physiological levels would be quite challenging, animals for each individual component of this cross already exists. Additionally, findings from recent genome-wide association studies (GWAS) suggest that after exclusion of the apolipoprotein E4 allele association, increased susceptibility to sporadic AD may be conferred by an accumulation of aberrations in genes involved in select pathways that affect brain lipid metabolism and inflammation [19,136]. It would, therefore, be informative to incorporate some of these recently identified new risk genes into the construction of future AD models.

While additional transgenic mouse models that more fully recapitulate the neuropathology of AD should be generated and characterized, additional consideration should be given to probing behavioral characteristics, in new models as well as in currently available models that are not associated with learning and memory. While there is a plethora of literature reports demonstrating nearly complete reversal of spatial memory impairments with numerous agents in various FAD transgenic mice, few reports actually document treatment-dependent changes in other behavioral domains [52]. Indeed characterization of anxiety phenotypes in FAD transgenic mice during aging or after pharmacological intervention is minimal and

Table 1. MicroPET studies in mouse models of Alzheimer's disease.

Ligand	End point	Model	Ref.
^{18}F-FDG PET	Cerebral glucose utilization	Tg2576	Luo *et al.* 2010, [102]
		Aged mice	Day *et al.* 2011, [103]
^{11}C-PIB	Amyloid Beta Deposition	APP23	Maeda *et al.* 2007, [97]
^{18}F-FDDNP		APP23	Higuchi *et al.* 2009, [104]
		Tg2576	Kuntner *et al.* 2009, [105]
^{18}F-THK523	Tau	P301L 4510	Fodero-Tavoletti *et al.* 2011, [106]
^{11}C-PK11195	Microglial activation	APP/PS1	Venneti *et al.* 2008, [107]
Fe- DAA1106		APP23	Maeda *et al.* 2007, [97]
^{11}C-AC516		APP23	Higuchi *et al.* 2009, [104]
		P301S	Higuchi *et al.* 2009, [104]
		APP23	Maeda *et al.* 2011, [108]
		P301S	Maeda *et al.* 2011, [108]

Table 2. MRI studies in mouse models of Alzheimer's disease.

End Point	Model	Ref.
Structure	PDAPP	Weiss *et al.* 2002, [109]
	Tg2576	Dedeoglu *et al.* 2004, [110]
	PDAPP	Song *et al.* 2004, [111]
	Tg2576	Sun *et al.* 2005, [112]
	PS2APP	Von Kienlin *et al.* 2005, [113]
	TASTPM	Maheswaran *et al.* 2009, [114]
	Tg2576	Luo *et al.* 2010, [115]
Amyloid plaques	APP/PS1	Jack *et al.* 2004, [116]
	Tg2576	Braakman *et al.* 2006, [117]
	Ts65Dn	Chen *et al.* 2009, [118]
	APP/PS1	Wengenack *et al.* 2008, [119]
	CRND8	Thiessen *et al.* 2010, [120]
	APP/PS1	Teipel *et al.* 2011, [121]
CAA	TGFB1	Lifshitz *et al.* 2011, [122]
	APP23	Beckmann *et al.* 2011, [123]
Functional		
fMRI	SAMP8	Zhang *et al.* 2007, [124]
CBV	J20	Moreno *et al.* 2007, [125]
CBV	Tg2576	Luo *et al.* 2010, [115]
CBF	APP/PS1 KI	Faure *et al.* 2009, [126]

SAMP: Senescence-accelerated prone mouse; TASTPM: APP(695(K595N, M596L)) X PS1(M146V).

even lacking for some models. These latter behavioral phenotypes represent a clearly under-investigated area of psychiatric disturbances that are associated with AD progression and one that might offer a more robust and potentially translatable behavioral endophenotype.

5.2 Greater use of small animal imaging

There is also a great need to expand our knowledge and understanding with respect to the utility of small animal imaging modalities. For instance, emerging evidence in humans suggests that measuring brain network activity may be an early and measurable endpoint in individuals that are genetically susceptible to AD [130,131] Relatively young individuals that are apoE4 carriers have enhanced brain network activity in the medial temporal lobe along with a concomitant decrease in connectivity to other brain regions [137]. Additionally, in elderly but otherwise cognitively normal subjects who have evidence of amyloid accumulation in these same brain regions, a lack of network connectivity has been reported [138]. Similar studies have not yet been reported in animal models over-expressing APP, alone or in combination with the human apoE4 allele.

5.3 Probing functional endpoints

Electroencephalographic (EEG) and sleep abnormalities are recognized early changes in AD patients and there is increasing interest in the use of quantitative EEG as a translational and noninvasive clinically relevant biomarker for studies in AD transgenic mouse models. Wang *et al.*, 2002, were the first to study changes in EEG activity in an AD mouse model and reported that APP/PS1 mice had reduced cortical theta activity and enhanced beta and gamma activity, but these changes were not age or amyloid plaque dependent [139]. By contrast, others have reported increased hippocampal theta power during wakefulness together with a decrease in delta frequencies in APP/PS1 mice (preceding amyloid plaque deposition) compared with wild-type and PS1-only mice [140]. The latter results are more analogous to the EEG patterns reported for AD patients. Further studies in a novel knock-in mouse model expressing mutations in APP, human tau and PS1 demonstrated that EEG anomalies paralleled impairments in long-term and short-term hippocampal plasticity but preceded cognitive deficits in recognition memory, spatial learning and sleep fragmentation [141]. These results indicate that the EEG endophenotype, characteristic of the prodromal stage of AD may be modeled experimentally with clinical translational validity. Additionally APP transgenic mice have demonstrated that even slight aberrations at select synapses may have profound effects on neuronal connectivity patterns resulting in overall hyperexcitability [57].

Loss of circadian rhythmicity in human AD subjects manifests in inefficient sleep and increased nocturnal activities [142]. Additionally, circadian rhythm disturbances

can manifest as exacerbated locomotion, anxiety, agitation and even delirium during periods just prior to sleep [143]. This 'sundowning' syndrome is thought to occur in AD subjects at the onset of neurodegeneration in the suprachiasmatic nuclei [144]. Moreover, elderly individuals tend to have a higher incidence of cataracts, macular or optic nerve degeneration limiting appropriate photostimulation of the circadian clock. The limited studies in FAD transgenic mice have quantified disruptions in circadian rhythm along with changes in the levels of anxiety-like behaviors that are diurnal. Treatment of older FAD transgenic mice with nightly doses of melatonin did not relieve the anxiety-like behaviors. However, in a separate study, passive immunization with an anti-Aβ antibody was able to normalize rapid eye movement sleep abnormalities supporting a hypothesis that Aβ peptides may have neurotoxic effects on the suprachiasmatic nuclei [145,146]. Given the tremendous burden the constellation of psychiatric behaviors and sundowning syndrome poses, more thorough investigations with various FAD transgenic mice are warranted especially given the potential to utilize circadian rhythm abnormalities as a translatable biomarker.

5.4 Understanding pharmacodynamic endpoints

In addition to expanding and incorporating functional endpoints in studies using preclinical models of AD, more emphasis should be placed on building and probing pharmacological endpoints that might serve as biomarkers during subsequent clinical development. Incorporation of target-related biomarker strategies early during a drug discovery campaign should enable an important assessment of how much target engagement will be required to drive a potential clinically meaningful endpoint. It is now a matter of routine to measure biochemical changes in Aβ and/or tau levels in brain homogenates in transgenic mice following administration of various agents. As an example, significant decreases in brain Aβ levels have been reported to occur following oral administration of potent GSIs to transgenic and wild-type mice at doses that translate into similar effects when administered to dogs [147,148]. Furthermore, the reduction in brain Aβ levels mirrored similar reductions in CSF from treated animals [148]. This concordance in the pharmacodynamic effects across preclinical animal models enables selection of doses with an appropriate safety margin for subsequent clinical trials. Although collection and measurement of various analytes in CSF is becoming a matter of routine in clinical studies, less frequent is the measurement of similar endpoints in CSF collected from transgenic mice. Important insights into the amount and duration of target inhibition that must be achieved in brain can be garnered from understanding how accurately the reductions in CSF levels of various Aβ species correlate to similar changes in brain homogenates. Although limited data using pharmacological intervention has been reported using agents that may alter the processing and/or appearance of various tau species, even interstitial fluid levels of tau can be measured in transgenic mice that express mutant tau isoforms and these changes also appear to change when increased neuropathology is apparent [149]. Within this framework, agents that are thought to modify phosphorylated tau and/or are believed to confer neuroprotection as a primary mechanism of action should result in changes in CSF phospho-tau levels, which may then be correlated to attenuation of frank neurodegeneration. Reliable measurements of these endpoints in animal models that have some face construct validity with regard to neuropathological phenotypes would enable investigators to link the difficult concept of measuring target engagement with a change in a pharmacodynamic endpoint that is related to disease progression.

5.5 Learning from recent Phase III failures

While significant limitations exist with fully recapitulating the entirety of AD clinical symptoms and neuropathology within the context of a transgenic mouse model, opportunities do exist to better evaluate and bolster a preclinical pharmacology package prior to the initiation of Phase IIb studies. While initial epidemiological studies suggested that nonsteroidal anti-inflammatory agent use could prevent or dampen the symptoms of AD, a well executed Phase III study designed to probe the potential clinical benefit of tarenflurbil (Flurizan, R-flurbiprofen) in AD subjects with mild-to-moderate disease failed to demonstrate any meaningful outcomes [150,151]. Retrospective analysis suggests that this lack of clinical efficacy could be directly attributed to the inability of compound to enter brain and engage the target at concentrations that would have affected changes in biomarkers. Indeed, in whole-cell assays, the concentration of compound required for the inhibition of Aβ42 generation was much higher than levels that could have been achieved centrally *in vivo* in animal studies and thus the exact basis of efficacy reported in the initial Phase II trial in humans is unclear [17,151].

Recent and substantial progress has also been made when utilizing pharmacokinetic/pharmacodynamic (PK/PD) modeling to probe the complexities between Aβ synthesis and clearance within different brain and peripheral compartments [147]. Some small-molecule GSIs have been observed to have long-lasting pharmacodynamic effects that cannot be explained by the availability of drug at the target. This latter observation may not be too surprising given that γ-secretase activity is embedded within the lipid bilayer of a cell. Accurate *in vivo* assessment using a semi-mechanistic model PK/PD model is required in order to predict human doses that will ultimately be tested in the clinical setting. Moreover, by applying sensitive ELISA measurements, accurate PK/PD modeling using GSIs administered to wild-type mice appropriately recapitulated the poorly understood rise in plasma Aβ levels. Additionally, an incomplete understanding and appreciation of other gamma secretase substrates that the GSI semagacestat may have inhibited probably contributed to the recent Phase III failure of this compound [20,24].

While structure–activity relationships along with physicochemical properties of small molecules can inform chemical design, construction of testable hypotheses and/or identification of a specific target/system will increase confidence in the therapeutic potential (**Figure 2**). Indeed, the generation and integration of knowledge as appropriate tools are applied to relevant animal models enables early construction of biomarker endpoints that further enhance the probability of technical success in the clinic. Additionally, appropriate and careful thought can be applied to patient selection strategies that may even be probed during the preclinical phase of discovery. Recent debate has centered around the need to test potential clinical candidates in populations that are at genetic risk for AD [152]. As a hypothetical example of this scenario, full evaluation of efficacious endpoints could be completed in transgenic mouse models that express the exact mutation as in the intended target population to ensure that an effective drug concentration will result in measurable clinical changes.

6. Expert opinion

Transgenic mouse models of AD have been utilized to probe novel mechanisms associated with AD pathogenesis. Several unexpected and novel findings that were first discovered using some of these same transgenic models have led to a new era of clinically testable hypotheses. Prior to the first active immunization approaches that were reported in PDAPP mice, vaccination against the neurotoxic Aβ peptide seemed unrealistic. Today, although still in the very early stages of clinical development, vaccination paradigms to prevent the onset of AD in certain defined populations are a real possibility. Similarly, prior to the first reported experiments utilizing passive administration of anti-Aβ antibodies to transgenic mice, it was unclear whether or not large IgG molecules could even enter the central nervous system. Today, preliminary reports in AD subjects administered two different anti-Aβ antibodies suggest that removal of long-lived deposited Aβ from brain is possible using passive immunization approaches. Furthermore, passive administration of anti-Aβ antibodies with very high affinity for soluble Aβ peptides also revealed novel insights into the possibility that the normal route of clearance of brain derived Aβ may occur by egress out of the central nervous system to the periphery for efficient degradation. Recent studies in AD subjects using SILK methodology has confirmed the initial observations in FAD transgenic mice suggesting that increasing brain Aβ burden alters the clearance rate of Aβ. This latter point opens up the possibility of probing therapeutic mechanisms for the prevention/treatment of AD that may not necessarily require delivery to the brain.

Significant insights into how hyperphosphorylated tau, which is a main constituent of NFTs, may interact with Aβ have been revealed by transgenic mice expressing various mutations associated with FTD. A recent and emerging theme that is evolving from studies using transgenic mice that overexpress genes associated with proteins prone to form brain amyloid (tau, Aβ , alpha-synuclein) is that the mutant or aberrant protein can 'template' endogenous protein causing sequestration of the non-mutant protein into pathological lesions. Furthermore, in addition to templating endogenous proteins, the hierarchically neuropathological phenotype that is observed suggests that a cell-to-cell transmission event may occur. While the concept of an extracellular transmission event is new and further work will be required to carefully dissect the exact mechanism(s) involved, these initial studies carried out in transgenic mice are exciting and offer novel routes for therapeutic intervention for AD as well as other neurodegenerative disorders.

While the use of transgenic mice that over-express mutations associated with FAD and FTD has provided facile models to study the dynamics of mutant proteins both biochemically and neuropathologically, there is a paucity of data probing the synaptic deficits that might occur. Recent studies in FAD transgenic mice are beginning to address this question; however, additional work is required. Since synaptic loss is significantly correlated to the cognitive decline associated with AD progression and is also an event that appears early during the course of the disease, a better understanding of the molecular and functional mechanisms that might be causative to these synaptic deficits would be advantageous. Similarly, the greater use of small animal imaging modalities such as functional MRI, ASL or MRS may provide novel insights into disease pathogenesis as well as provide translatable endpoints that could be directly monitored and implemented into clinical trial design.

Although GWAS have identified new loci that are associated with sporadic AD, the most robust susceptibility allele associated with late-onset AD remains the $\varepsilon 4$ allele of the apolipoprotein E (apoE4) gene. The association of apoE4 with AD reaches a GWAS significance of $\sim p < 10^{-100}$ and validates the initial observations that apoE4 (even in combination with apoE3) confers a greater disease risk at a younger age. Despite this convincing genetic association to AD, the biological cause(s) of this susceptibility remain unclear. Clearly, more effort and investigation utilizing transgenic mouse models are needed to further define and identify relevant and tractable apoE4-dependent pathways that may be amenable to drug discovery efforts. Additionally, GWAS efforts have also identified other loci that warrant further investigation in transgenic mouse models. Although conferring only minimal increased risk for disease, generating transgenic mice with these susceptibility variants should yield novel insights. As our ability to probe more sophisticated measures of function in transgenic mouse models increases and our biochemical assays become more sensitive, it may not be as critical to generate transgenic mice that vastly over-express mutant proteins. Subtle but reproducibly consistent changes in synapse function conferred by a hypothetical 'humanized' transgenic mouse may offer more insights and perhaps even greater potential for probing relevant AD mechanisms.

While early results of dramatic reductions in brain Aβ levels as well as robust improvements in spatial learning observed

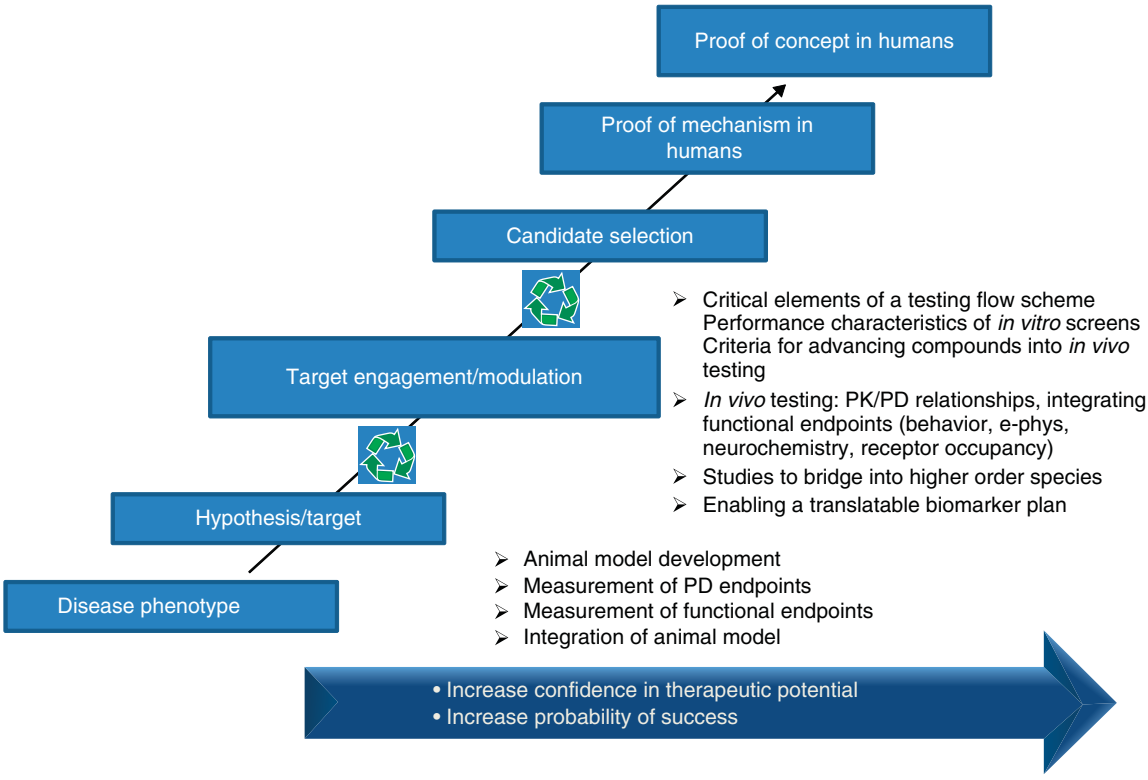

Figure 2. Increasing the probability of clinical success requires iteration and integration of project flow scheme activities beginning with the identification and characterization of an appropriate animal model through clinical biomarker development.

in FAD mice administered various Aβ lowering agents are exciting, it will be imperative to improve our understanding of PK/PD relationships in the context of relevant PD endpoints. This is especially important given recent regulatory guidance that mandates the investigation of amyloid-related imaging abnormalities for Aβ-lowering agents in preclinical animal models. Probing the ability of various Aβ-lowering strategies to prevent rather than reverse the accrual brain Aβ in various FAD transgenic mice has hampered our ability to appropriately define the level of target engagement that may be required to drive a meaningful clinical outcome in a safe manner.

Recently several failures have dampened overall enthusiasm for the possibility of effectively prosecuting potential therapies for AD. Learning from these failures must be implemented to avoid future disappointments. As we gain more knowledge about the heterogeneous genetic and environmental causes of AD, we must do everything possible to ensure that we have adequately investigated and set stringent criteria, for the pharmacodynamic, pharmacokinetic and functional endpoints in our preclinical animal models. In so doing, we not only increase the probability of clinical success but also increase our ability to test a number of hypotheses more accurately – a clear imperative given that the most effective therapy for AD subjects will probably require intervention at more than one target.

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Declaration of interest

K Bales is an employee of Pfizer.

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