The Amyloid Cascade Hypothesis: are we poised for success or failure?

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The first description of Alzheimer's disease (AD) was made in 1907 by Alois Alzheimer (Alzheimer 1907), although other contemporary physicians had made similar, and rather more complete, assessments of the neuropathological changes present in the AD brain (Fischer 1907). Our knowledge of AD has increased dramatically and continues to accelerate. This year is 25 years after the publication of a series of papers that, in various ways, articulated the amyloid cascade hypothesis (ACH) for AD (Hardy & Allsop 1991, Beyreuther & Masters 1991, Selkoe 1991, Hardy & Higgins 1992). This review will cover some familiar territory, but we shall also place the ACH into a wider context, compare it with other hypotheses for AD, explore the evolution of the hypothesis to encompass new findings, and determine, irrespective of the merits of the hypothesis itself, whether it has been useful for the research field, both in academia and in industry. Finally, we shall review how the ACH has led to a number of therapeutic approaches, all of which have, to date, failed to reach their primary efficacy end-points in clinical trials and reflect upon what the future may hold.

The hypothesis

The major support for the ACH comes from the combination, and interdigitation, of pathophysiology and human genetics. The origins of the ACH lie in the sequencing of the amino acid sequence of Aβ extracted from cerebral blood vessels (Glenner & Wong 1984b) and then brain parenchyma (Masters *et al.* 1985) of post mortem brains from AD patients. This led to the identification and sequencing of the amyloid precursor protein (APP) gene (Kang *et al.* 1987) that encodes the holoprotein from which the Aβ peptide is excised by the sequential action of β-amyloid cleaving enzyme (BACE) to release the N-terminus of Aβ (Hussain *et al.* 1999, Sinha *et al.* 1999, Vassar *et al.* 1999, Yan *et al.* 1999, Lin *et al.* 2000) and γ-secretase that cleaves at the C-terminus (De Strooper *et al.* 1998, Wolfe *et al.* 1999b). γ-secretase is a multi-protein complex comprised of Presenilin (PS)1 or PS2; aph1a or aph1b; Pen2 and nicastrin (for review, see (De Strooper *et al.* 2012)) with the PS proteins incorporating the enzyme's active site aspartyl resides (Wolfe *et al.* 1999a, Wolfe et al. 1999b). Although other publications were articulating similar viewpoints, (Hardy & Allsop 1991, Selkoe 1991, Beyreuther & Masters 1991), the ACH was expounded most trenchantly in 1992 (Hardy & Higgins 1992). In this admirably brief paper, the deposition of the Aβ peptide was portrayed as an upstream event in the evolution of AD, leading to cell death and/or the development of neurofibrillary tangles (NFTs) (hyper-phosphorylated, insoluble tau aggregates) via elevation of intracellular calcium ion levels. A critical component of the ACH comes from

human genetics, where a family history of early onset AD led to a linkage analysis study that revealed a mutation resulting in a V717I amino acid change in the *APP* gene just C-terminal to the Aβ peptide sequence (Goate *et al.* 1991). This seminal work revealed that a single mutation resulted in early onset AD that was pathologically identical to sporadic, late onset AD. The potential for a genetic cause for AD had been made previously: in 1984, Glenner and Wong made the prescient observation that because the Aβ peptide deposited in the cerebrovasculature of elderly Down's syndrome (Trisomy 21) subjects was identical (bar an experimental error at one position) to the 21 amino acids that were sequenced from sporadic AD (SAD) patients, a genetic defect on chromosome 21 was likely to be a cause of AD (Glenner & Wong 1984a).

Aβ/amyloid pathology is seen in all AD patients, by definition, although its relationship to cognitive decline is unresolved (Arriagada *et al.* 1992, Delacourte *et al.* 1999, Naslund *et al.* 2000). There are now hundreds of mutations to three genes, *PSEN1*, *PSEN-2* and APP that can cause early onset familial AD (FAD) (http://www.alzforum.org/mutations). These mutations all have the effect of increasing the amount of C-terminally extended Aβ peptides, or the ratio of aggregatory, longer forms of Aβ to shorter, more soluble forms, or to increase the aggregatory properties of the Aβ peptide directly (Citron *et al.* 1992, Suzuki *et al.* 1994, Scheuner *et al.* 1996, Borchelt *et al.* 1996, Duff *et al.* 1996, Bentahir *et al.* 2006, Hori *et al.* 2007, Inayathullah & Teplow 2011, Ni *et al.* 2011, Chavez-Gutierrez *et al.* 2012, Szaruga *et al.* 2015). The longer forms of Aβ are extended at the C-terminus with hydrophobic amino acids that greatly increase the propensity for aggregation (Jarrett *et al.* 1993). At this time, several groups made the critically important discovery that Aβ was a regular secretion product from cells that expressed the *APP* gene rather than being produced solely under unusual or physiologically stressful conditions (Shoji *et al.* 1992, Haass *et al.* 1992, Seubert *et al.* 1992): this breakthrough enabled the development of cell-based assays to screen for compounds to inhibit Aβ production. Ultimately, cell based assays were used in the discovery of γ-secretase inhibitors: competitive (Shearman *et al.* 2000, Esler *et al.* 2000, Li *et al.* 2000); non-competitive (Dovey *et al.* 2001, Lanz *et al.* 2006)) and modulator compounds (Weggen *et al.* 2001). The FAD mutations all localize to proteins involved in the production and properties of the Aβ peptide. With respect to SAD, the greatest genetic risk factor is *Apolipoprotein (APOE) 4* (Corder *et al.* 1993). The human population has three major alleles of *APOE – APOE2*, *APOE3* and *APOE4* (Nickerson *et al.* 2000). One allele of *APOE4* increases the risk of AD four-fold compared to an *APOE3*/*APOE3* genotype; two copies of *APOE4* increases the risk approximately 12-fold and the *APOE2* allele reduces risk compared to *APOE3* (Verghese *et al.* 2011). While the biology of ApoE is undoubtedly complex (Liu *et al.* 2013, Holtzman *et al.* 2012), there is very strong evidence that show that ApoE is required for the deposition of amyloid from preclinical (Bales *et al.* 1999, Holtzman *et al.* 2000, Holtzman *et al.* 1999) and clinical (Schmechel *et al.* 1993, Jack *et al.* 2015)studies. Moreover, the increased probability of having brain amyloidosis matches the ApoE isoform dependent risk of succumbing to AD. Additional evidence for the primacy of the role of Aβ in AD comes from the A673T mutation (Jonsson *et al.* 2012) that significantly protects against AD. This mutation (Figure 1) decreases both the production and aggregatory properties of Aβ (Maloney *et al.* 2014, Benilova *et al.* 2014).

In summary, the ACH accommodates a wide range of data that we have on AD into a coherent hypothesis. It is worth articulating three tenets that have to be true if ultimately we will refute the null hypothesis.

1. The parenchymal deposition of the Aβ peptide is important pathophysiologically.
2. Aβ peptide deposition occurs prior to the frank neuronal and synaptic loss that is the hallmark of AD.
3. The evidence from mutations that cause FAD is informative and relevant to SAD.

Taking each in turn, (1) is to a certain extent a matter of semantics. As a definite diagnosis of AD relies on neuropathologic evidence revealing Aβ plaques and NFTs, presence of deposited Aβ peptide is by definition required. However, the role of deposited Aβ peptide as being the preeminent disease-causing Aβ species has been brought into question by the burgeoning literature on smaller molecular weight oligomeric Aβ (oAβ) (see later). Thus, it could be argued that the deposition of Aβ is irrelevant to the disease process as such, or at least of much lesser importance. (2) is likely true although now open to a different interpretation. The ACH as originally conceived placed amyloid as upstream of tau pathology, and yet detailed neuropathological studies have shown that tau pathology is present from a very early age in some people and in all cases precedes amyloid pathology (Braak *et al.* 2011). However, correlative studies have shown that amyloid pathology likely drives tau pathology from restricted allocortical sites to proliferate throughout the cortex leading to widespread neuronal loss (Price & Morris 1999). (3) is still a matter of significant debate, but it seems very likely that the familial and sporadic forms of the disease are identical in all major respects. For example, the neuropathology of SAD and FAD are indistinguishable from each other (Nochlin *et al.* 1993, Lippa *et al.* 1996). Thus, for the diseases to be fundamentally different, it would be necessary to explain why the pathological changes in a FAD brain lead to a different disease process than in a SAD brain. Furthermore, as stated previously, the greatest genetic risk factor for SAD is carrying an *APOE4* allele which also brings forward the age of onset for the disease (Corder et al. 1993). In very large cohorts of FAD patients that all carry the same PSEN1 mutation, it has been demonstrated that the *APOE4* allele also brings forward the age of onset (Pastor *et al.* 2003). If SAD and FAD were very different diseases, it would be unlikely that they would both be subject to the same genetic modifier.

Before exploring some of the deficiencies in the ACH, it is worth briefly considering alternative hypotheses that have been posited for the onset and progression of AD.

Mitochondrial cascade hypothesis

The mitochondrial cascade hypothesis (MCH) posits that age-related mitochondrial dysfunction ultimately leads to the pathology and symptomatology of AD (Swerdlow & Kahn 2004, Swerdlow *et al.* 2014). It also attempts to resolve the difficult question of whether SAD is a usual, but not necessarily universal, consequence of brain aging. Indeed, in very elderly cohorts the prevalence of AD pathology may exceed 50% (Polvikoski *et al.* 2001). There is little doubt that there is evidence of mitochondrial damage in the brains of people suffering from AD (Lin & Beal 2006). Furthermore, fluorodeoxyglucose positron emission tomography (PET) imaging has revealed deficits in the AD brain quite early on in the disease process, which as a marker of oxygen uptake is likely affected due to alterations in mitochondrial function (Jack *et al.* 2012). Also, there is abundant evidence of increases in free radical damage in AD brains that again might result from dysregulated mitochondrial function (Sonnen *et al.* 2008). Some of the experimental support for the MCH comes from the use of cybrids which is where a cell line is treated with ethidium bromide to block mitochondrial DNA replication to from a 'p0' cell.

These cells are then co-cultured with platelets from a human host (e.g., with AD) in the presence of polyethylene glycol that induces the transfer of platelet mitochondria into the p0 cells to form a cybrid (King & Attardi 1989) – a cell containing mitochondria from a different cell. Experiments with AD cybrid cells have shown an increase in Aβ production (Khan *et al.* 2000) and reactive oxygen species (Cardoso *et al.* 2004). The MCH uses such data to suggest that a mitochondrial deficiency in AD brains underpins an increase in Aβ production. In the MCH, Aβ is a biomarker for brain aging, but not a cause of AD as such.

In our opinion, the MCH fails to answer some key questions. Despite very large genome wide association studies (GWAS), no genes that encode mitochondrial proteins or proteins involved in bio-energetics have been found (Lambert *et al.* 2013). Some of the evidence in support of the MCH comes from cell biological systems that are significantly manipulated and might not reflect the human pathophysiological situation. For example, demonstrating that cybrid cell cultures show an increase in Aβ production does not reflect the situation in SAD, or, in most cases, in FAD, where the majority of mutations alter the ratio of Aβ metabolites or their propensity to aggregate, but do not increase the absolute amount – indeed, many reduce overall Aβ production (Chavez-Gutierrez *et al*. 2012, Szaruga *et al*. 2015). The MCH fails to account for the A673T mutation in APP (Jonsson *et al*. 2012) that has been demonstrated to protect against SAD by reducing Aβ production modestly and reducing its propensity to aggregate (Jonsson *et al*. 2012, Benilova *et al*. 2014, Maloney *et al*. 2014). One of the tenets of the MCH, that AD is largely a disease of aging, can also be questioned on several levels. For example, none of the genes that causes FAD is known to play a role in aging, and none of the mutations that causes progerias results in accelerated AD pathology (Nelson *et al.* 2011). Critically, the MCH does not articulate how mitochondrial dysfunction leads to the full panoply of AD pathology, which is something it shares in common with the ACH.

Dual Pathway Hypothesis.

The dual pathway hypothesis (DPH) (Small & Duff 2008) seeks not to refute the ACH as such, but more to refine it especially insofar as SAD is concerned. Part of the motivation for so doing is based on the failure of drugs targeting amyloid (amyloidocentric) to provide therapeutic benefit. In fact, from the time the DPH was published, the litany of failure for amyloidocentric drugs has significantly worsened (Karran & Hardy 2014, Cummings *et al.* 2014). Post mortem data from patients that participated in the Phase 1 AN1792 study (Nicoll *et al.* 2003, Holmes *et al.* 2008, Paquet *et al.* 2015), where amyloid was apparently cleared from the brains of patients but cognitive decline proceeded unabated, is used to support the view that amyloid deposition in SAD does not necessarily drive other pathologies, as is implicit in the ACH. The DPH posits that there might be upstream factors that are able to drive both the major pathologies, amyloid and tau, such that treatments downstream of these factors will not ultimately provide therapeutic benefit. The data supporting potential upstream mechanisms includes, for example, ApoE4, which might act to increase Aβ deposition via reduced clearance and increase tau phosphorylation via low-density lipoprotein receptor related protein (LRP)5 and LRP6 signaling to activate glycogen synthase kinase 3β activity.

However, and as pointed out by the authors, the data supporting ApoE4 mediating its effects via Aβ are very compelling and more substantive than are the links to tau pathobiology. Further, the phenotypic

effect of ApoE4 to bring forward the age of onset for AD is present in both SAD and FAD (Pastor *et al*. 2003), strongly implying that SAD and FAD are similar disease processes. Finally, from neuropathological and clinical correlations, tau pathology is better correlated with both neuronal loss and symptomatology (Arriagada *et al*. 1992, Gomez-Isla *et al*. 1996). If ApoE4 was driving both pathologies, one might anticipate that ApoE4 would bring forward the age onset of disease and accelerate disease progression: while there is excellent evidence for the former, there are little data to support the latter. Nevertheless, the DPH seeks to resolve the disconnection between amyloid and tau pathology and to explore the upstream triggers for disease in SAD, which is totally unexplained by the ACH.

The Metabolism Hypothesis

The metabolism hypothesis (MH) has its origins the work of Hoyer and colleagues (Hoyer *et al*. 1988, Hoyer 1991) who believed that the underlying cause of AD was cerebral glucose hypometabolism. To investigate this phenomenon they developed a rat model that involved injecting streptozotocin intracerebroventricularly into rats (Lannert & Hoyer 1998). This resulted in decreased glucose/energy brain metabolism together with learning and memory deficits. Streptozotocin is an agent widely used in the diabetes field to destroy β pancreatic cells in rats to produce an insulin-dependent diabetic state. Since these early experimental approaches, a significant body of evidence has grown that indeed establishes that insulin signaling in the brain is significantly impaired. For example, many of the mRNA transcripts encoding key elements of the insulin signaling pathway are significantly depressed in multiple regions of the AD brain (Steen *et al*. 2005) leading to the description that AD is 'Type 3 Diabetes'. Proponents of the MH argue that insulin signaling is required for preserving synaptic connectivity and may play a role in neuronal stem cell activation and neuronal 'resilience'. Some very interesting preclinical work has been performed with incretin mimetics such as liraglutide, which when administered via intraperitoneal injection to APP Swe/PS1ΔE9 mice (25nmol/kg od) was shown to reduce Aβ plaque load significantly (McClean & Holscher 2014). There are also data that show that Aβ oligomers (oAβ) bind to and antagonize various components of the insulin signaling pathway and that this may lead to an increase in activity of GSK-3β, a known tau kinase (Morgen & Frolich 2015). Furthermore, as stated earlier, imaging studies using fluorodeoxyglucose PET brain imaging, which is a measure of glucose uptake and neuronal activity, reveals deficits very early on in the clinical course of AD patients (Jack *et al*. 2011).

It is difficult to extrapolate the findings from the streptozotocin rat experiments to AD: there is very little face or construct validity given the relatively non-specific mechanism of action of the toxin. The role of oAβ to provoke insulin signaling abnormalities is rather weak, mainly because the role of oAβ themselves is controversial (Benilova *et al*. 2012). It is difficult to assign a primary role of insulin signaling abnormalities in disease causation as currently there are no data from GWAS studies to support such a role (Lambert *et al*. 2013), and the data that place insulin signaling abnormalities upstream of tau and amyloid pathology are not yet compelling. It may be the case, however, that insulin signaling pathways are adversely affected due to the AD disease process itself, and in general this hypothesis has merit because it provides a number of testable hypotheses and avenues for therapeutic intervention. Indeed, there have been a number of small clinical experiments where insulin has been delivered directly intra-

nasally into the brain with promising results (Bedse *et al.* 2015, Craft *et al.* 2012), leading to a much larger clinical trial that is underway (ClinicalTrials.gov Identifier:NCT01767909).

Cell-Cycle Re-entry Hypothesis

The cell-cycle re-entry hypothesis (CCRH) might be considered a particular form of a more general hypothesis that posits that an age-related increase in DNA damage in neurons is responsible for neurodegenerative disease (Chow & Herrup 2015). Neurons are post-mitotic cells and therefore need to sustain their genomic integrity for life.   Neurons might also be subject to significant stressors during life: being cells with a very high energy requirement, the potential for DNA damage via reactive oxygen species is significant coupled with the errors that can result from gene transcription (Poduri *et al.* 2013, Lodato *et al.* 2015). The first indications of a potential role for aberrant cell cycling in the brain came from observations demonstrating that mitogen kinases had increased expression in the AD brain (Arendt *et al.* 1995) and that specific antibodies raised to paired helical filaments (PHFs) of tau purified from AD brain cross-reacted with epitopes in dividing cells (Vincent *et al.* 1996). Further work revealed that driving primary, differentiated neurons to divide by infecting with oncogenes c-myc and ras resulted in DNA duplication and increases in both anti-phospho-tau immunoreactivity and AD-like abnormally folded tau epitopes (recognized by ALZ50 antibody) (McShea *et al.* 2007). However, the cells did not enter mitosis, suggesting that the cells get blocked at the G2/M transition. The c-myc oncogene was also conditionally expressed in frontal cortex using CaMKII-tTa transgenic mice crossed to tet-o-Myc transgenic mice (Lee *et al.* 2009). c-Myc expression was initiated by removing doxycycline from the diet. The bigenic mice showed markers of cell cycle activation, DNA replication, hippocampal neuronal loss and cognitive behavioral deficits. Surprisingly, given previous work, evidence for abnormal tau phosphorylation was not presented. In post mitotic neurons, DNA repair is afforded by a variety of mechanisms which if disrupted can result in a number of serious neurological abnormalities including neurodegeneration (McKinnon 2013) but these are mostly early onset developmental conditions. Linking frustrated mitotic cell division to the range of neuropathology evident in AD is currently challenging. While a plausible link to tau pathology can be made, the data suggesting a role for cell cycle re-entry in Aβ deposition is not substantial. Further, there is currently no human genetic evidence from GWAS studies that support a role for aberrant cell-cycle re-entry (Lambert *et al*. 2013), and it is difficult to reconcile the effect of the A673T APP mutation to protect against AD into this schema (Jonsson *et al*. 2012).

Vascular hypothesis

The vascular hypothesis (VH) was originally based on the neuropathological observations that the AD brain has a disorganized and much reduced capillary and vascular network (Fischer *et al.* 1990, de la Torre & Mussivand 1993). There is now a significant body of evidence that supports an important role for the brain's vascular system in AD (de la Torre 2004, Marchesi 2011). Some of the known risk factors for AD include hypertension in midlife and diabetes in late-life (and probably in midlife) both of which have significant vascular morbidities (Prince *et al.* 2014). A study of the localization of thioflavine T-staining amyloid plaques in a range of dementias (AD, Pick's disease, Guam amyotrophic lateral

sclerosis/parkinsonian dementia complex, Down syndrome, dementia pugilistica and prion disease) revealed significant reductions in the microvasculature and some co-localization of blood vessels and plaques (Buee *et al.* 1994). This latter aspect was investigated more thoroughly and the co-localization of haem-rich deposits, amyloid plaques and blood vessels was demonstrated in AD and Down's syndrome brains (Cullen *et al.* 2006). A very thorough investigation of this relationship was performed using the TG2576 and the PSAPP transgenic mouse models that both develop parenchymal amyloid deposition (Kumar-Singh *et al.* 2005). These studies revealed that 85-95% of dense core plaques were either centered on, or adjacent to, vasculature vessel walls. Interestingly, the same was not true for diffuse amyloid. However, in both these models the Aβ composition of the plaques is dominated by the Aβ40 species, unlike AD plaques which are made predominantly of Aβ42 (Welander *et al.* 2009). Aβ40 is more soluble than the Aβ42 and more likely to be able to be trafficked to the vasculature via bulk interstitial fluid flow. However, these studies cannot confirm a temporal relationship – do amyloid plaques result in vascular damage, or does vascular damage result in plaques? To investigate this aspect, the generation of plaques was investigated using electron microscopy and the earliest signs of fibrillary Aβ appeared to form in the perivascular space. This work relates importantly to the clearance of Aβ from the brain: clearly, if extracellular concentrations of Aβ are kept low, the potential for aggregation will likely be diminished. A detailed analysis of brain clearance mechanisms is beyond the scope of this review (see (Weller *et al.* 2008, Tarasoff-Conway *et al.* 2015)) but there have been some recent important developments. A clearance pathway – the glymphatic system – has been described for the first time (Iliff *et al.* 2012) that is part of the Aβ clearance mechanism. The glymphatic (glial-lymphatic) system consists of perivascular conduits formed by glial cell end-feet. Interestingly in the context of AD, these glial processes are also a site of ApoE expression. Fluid is believed to be impelled through the glymphatic system at least in part by the pulsatile contraction of smooth muscle cells in the vasculature, providing a potential mechanistic link with the vascular system. There are several aspects of the VH that integrate multiple elements of features of AD: the known co-morbidities, the important role in Aβ clearance and providing potential sites for initial Aβ deposition. However, what is difficult to determine is primacy of effect. Does a vascular insufficiency leading to impaired Aβ clearance, or local vascular damage, provide the appropriate local environment for amyloid deposition, or does amyloid deposition result in vascular damage: or can it be both?

Aβ Oligomer Hypothesis

The Aβ Oligomer Hypothesis (ABOH) is a variant of the original ACH that currently has significant momentum (Walsh & Selkoe 2007). The ABOH posits that small molecular weight oAβ represent neurotoxic agents that cause synaptic damage in AD. There are significant attractions to the ABOH, principle amongst which is a potential resolution of a major conundrum of AD research: amyloid plaques do not correlate in terms of their amount, nor brain regional location, with AD symptomatology or neuronal loss (Gomez-Isla *et al.* 1996, Delacourte *et al.* 1999). Indeed, it is difficult to visualize how very insoluble, relatively inert protein deposits are able to exert a damaging effect on the brain. Thus, oAβ might act at a distance from plaques and mediate toxic effects. There are wealth of data, beyond the scope of this review, that together provide a large body of supportive data for the ABOH. These include: the manufacture of various aggregated forms of Aβ in vitro, using Aβ42, Aβ40, ratios, and modified

forms thereof at supra-physiological concentrations to make oAβ; the profiling of these using analytical techniques such as size exclusion chromatography, sodium dodecyl sulfate - poly acrylamide gel electrophoresis gels (SDS-PAGE) and EM imaging; the treatment of in vitro neuronal cell cultures with (usually) supra-physiological concentrations to induce neuronal cell distress; the investigation of oAβ to affect a number of important neuronal receptors (eg, Insulin R, nicotinic receptors); the treatment of brain slice preparations to induce electrophysiological changes such as reduction or abolition of LTP; the injection of oAβ into rodent brains to induce impaired cognitive functioning (Haass & Selkoe 2007, Ferreira & Klein 2011, Koffie *et al.* 2011, Mucke & Selkoe 2012, Hayden & Teplow 2013, Hefti *et al.* 2013, Viola & Klein 2015). Also, there have been attempts to categorize oAβ in APP transgenic mice using anti-oAβ conformational antibodies (Liu *et al.* 2015). The ABOH, if true, would also have a profound effect on clinical development, as it could mean that therapeutics that are targeting amyloid plaque would not be efficacious, and indeed might be positively deleterious if by disaggregating plaques they released, or created favourable conditions for, increased levels of oAβ. Despite these data, and significant support in the AD research field generally, there a number of fundamental questions that remain to be answered with respect to the ABOH (Benilova *et al2012*). Hepler and colleagues (Hepler *et al.* 2006) have shown convincingly that aggregated Aβ runs aberrantly in size exclusion chromatography, mainly because monomeric Aβ does not perform as a solvated sphere. Further, these workers have shown SDS-PAGE cannot be used to resolve different Aβ species unequivocally, and also that the appearance of oAβ in electron microscopy is heavily dependent on the properties of the surface upon which the Aβ aggregates are dispersed. Thus, many analytical procedures routinely used in the field are confounded. The interpretation of neuronal cell death induced by oAβ in vitro is also problematic. Often, such experiments use neuronal cell lines or primary rodent neurons to demonstrate Aβ-mediated cell distress and death, sometimes in a manner that discriminates between different oAβ forms (Ono *et al.* 2009, Ahmed *et al.* 2010). However, in APP transgenic mouse models, which reveal plaque pathology that is very similar to that seen in AD, neuronal loss is usually completely absent (Irizarry *et al.* 1997a, Irizarry *et al.* 1997b). Thus, human Aβ can be toxic to rodent neurons in vitro, but often not in vivo.

The most challenging of these questions is to provide unequivocal evidence of their existence, and role, in the AD brain. Attempts to measure oAβ in intercellular fluid in brain parenchyma of transgenic mice that develop Aβ plaque pathology have been very technically challenging (Hong *et al.* 2014) as have measurements of oAβ in cerebrospinal fluid from AD patients. Indeed, recent work with sensitive assays has failed to reveal a difference between oAβ concentrations in AD versus controls (Yang *et al.* 2015). These studies have been confounded by a lack of a biophysical definition of an oligomer and no standard preparation of a single species with which to calibrate and control assays. It is important, in this context, to distinguish between *measuring* oAβ, and *extracting* oAβ. There are multiple papers that use various techniques to extract a range of oAβ species from AD brains that can be subsequently revealed on denaturing SDS-PAGE, followed by Western blotting using anti-Aβ antibodies (Ward *et al.* 2000, Upadhaya *et al.* 2012). However, these techniques likely create oAβ species due to the physicochemical properties of the Aβ peptide as discussed earlier. Leaving aside these issues, perhaps the most compelling evidence for a role of oAβ could be inferred from human genetics - indeed, the V717I mutation that causes FAD provided the cornerstone for the amyloid cascade hypothesis (Goate *et al*. 1991). The APP gene has a wide range of different mutations (http://www.alzforum.org/mutations) that cause FAD and/or congophilic amyloid angiopathy (Figure 1). These can be either N-terminal or C-

terminal to, or within, the Aβ42 peptide. A consideration of the latter is particularly instructive because these are unlikely to affect the production of Aβ or the ratio of long to shorter forms that has been shown previously to be important in age of disease onset (Duering *et al.* 2005, Kumar-Singh *et al.* 2006). Mutations within the Aβ42 peptide are more likely to affect the folding of the peptide, although other aspects may also be affected, such as clearance from or degradation within the brain. However, a mutation that locked the Aβ peptide in an oAβ from, or greatly inhibited the formation of amyloid fibrils so as to increase the proportion of oAβ, would add significant weight to the ABOH.

The amino acid position 2 of the Aβ sequence (with the N-terminal Aβ aspartic acid = 1) is particularly interesting as it has both protective (A2T) (Jonsson *et al.* 2012) and disease-causing (A2V) mutations (Di Fede *et al.* 2009) (Figure 1). A2T results in a 50% reduction in Aβ production when over-expressed in primary mouse neuronal cells, which would equate to a 25% reduction in the heterozygotic state (Benilova *et al*. 2014). A2V, found as an autosomal recessive mutation in humans, approximately doubles production of Aβ by rendering APP a better substrate for β-secretase. However, both mutations also have effects on the aggregatory properties of the Aβ peptides that were revealed using synthetic Aβ40 preparations. Effects on Aβ42 were not informative for largely technical reasons: at the concentrations of Aβ used in these experiments, Aβ42 forms amyloid fibrils very rapidly compared to Aβ40 making dissection and measurement of aggregation very challenging. The protective A2T mutation increased the lag phase prior to aggregation and the change in Gibbs free energy compared with wild type was lower, demonstrating the reduction in aggregation. The A2V mutation almost abolished the lag phase, such that fibrillar amyloid was being formed almost immediately. Interestingly, antibodies that have been reported to be conformational, 'oAβ specific' (A11 and OC) (Kayed *et al.* 2007) failed to recognize either mutant Aβ in the soluble phase post-fibrillization despite a range of small Aβ species being visualized using electron microscopy. This finding rather casts doubt on the validity of these antibodies to measure oAβ specifically. Similar work was published using Aβ42 peptides (Maloney *et al*. 2014). Hori and colleagues (Hori *et al*. 2007) studied the effects of the H6R, D7N and E22G mutations. All the mutations increased the rate of formation of fibrillary amyloid, but this was mediated differently.   In the case of E22G, both seeding/nucleation and amyloid fibril elongation was increased significantly compared to wild type Aβ. This led to increases both in protofibril and fibril formation. The effects of the H6R and D7N mutations were to increase significantly amyloid fibril enlongation rate – that is, the rate at which monomers are added to the growing amyloid fibril. However, this led to very significant reduction in the concentration and residence time of oAβ species: presumably, the addition of monomers to existing amyloid fibrils was energetically more favourable than the formation of oAβ and protofibrils.

The ΔE22 mutation initially caused significant excitement in the field because the first reports suggested that the phenotypic effect was to promote oAβ formation and prevent Aβ fibril formation (Tomiyama *et al.* 2008). However, subsequent work revealed that this was likely an assay artefact (Inayathullah & Teplow 2011). The effects of the ΔE22 mutation are two-fold: firstly, there was an increase in the formation of oAβ species. However, this effect was dwarfed by an extraordinary increase in the propensity of the mutant to form β-pleated sheet fibrillar amyloid that was ~400-fold faster than wild type.

A comparative study of the A21G, E22G, E22Q, E22K and D23N mutations (Ni *et al*. 2011) showed that all of these species were able to form β-pleated sheet amyloid fibrils. With the exception of A21G, all of the

mutants decreased the lag phase for the formation of Aβ fibrils, some of them dramatically (E22Q, D23N). A21G resulted overall in an increase in total fibrillization, as did E22Q. It is important to note that these mid-domain changes may have other effects as well: for example, these mutations can cause cerebral amyloid angiopathy as well as AD, implying changes in clearance mechanisms (Van Broeckhoven & Kumar-Singh 2006, Zhang-Nunes *et al.* 2006).

In conclusion, the effects of the mid-domain Aβ mutations on aggregation do not provide data in support of the OABH, although caution is warranted in extrapolating from very artificial biochemical systems to the complexity of the human brain. Nonetheless, if anything the mid-domain mutations tend to increase the propensity of Aβ to form amyloid rather than oAβ. A compelling role for oAβ-type species is in the seeding of amyloid plaques. A wealth of careful and compelling science from Walker and Jucker, and others, have unequivocally demonstrated that a soluble Aβ species is able to seed Aβ plaque deposition (for review, see (Walker & Jucker 2015). Furthermore, these species are very stable, long lived and prion-like (Ye *et al.* 2015). The seeding of Aβ plaque in humans has also recently been postulated as a consequence of the treatment of humans with extracts from pituitary glands (Jaunmuktane *et al.* 2015) or with dura mater grafts (Frontzek *et al.* 2016) although caution must be exercised in interpreting these data given the very heterogeneous nature of the implanted tissues.

For each of the hypotheses considered here, including the ACH, rejection of the null will require firstly, the development of a specific therapeutic approach that addresses a key mechanism that is predicted by the hypothesis to be pathological, and secondly, the demonstration that the therapy has efficacy in a placebo-controlled, randomized clinical trial in AD patients. Also, it is important to distinguish between the causes of AD, and the consequences of the AD process. It is likely that many of the processes that are featured in the various hypotheses previously considered do play a role in AD, but they do not initiate the disease.

A version of the ACH hypothesis is provided (Figure 2) that we believe reflects, parsimoniously, the current state of knowledge. There are still a significant number of gaps in the ACH, many of which have been attacked by detractors of the hypothesis (Herrup 2015) and that we will address where possible.

Disease initiation

In SAD and FAD we do not know what initiates Aβ deposition. The ACH has sometimes been interpreted to suggest an increase in Aβ production results in AD, and indeed there are data that support this view (Potter *et al.* 2013) but in fact the majority of FAD mutations do not result in an increase in Aβ production, but an increase in the ratio of the longer to the shorter forms of the Aβ peptide (Citron *et al.* 1992, Suzuki *et al.* 1994, Scheuner *et al.* 1996, Borchelt *et al.* 1996, Duff *et al.* 1996, Bentahir *et al.* 2006, Chavez-Gutierrez *et al.* 2012, Szaruga *et al.* 2015). Also, Aβ is not present at a concentration in interstitial fluid at which it can spontaneously aggregate, so it must be a facilitated process. However, there is compelling data that very low abundance, stable, Aβ conformers can seed Aβ plaques as previously discussed. This opens the possibility that a very discrete, local increase in Aβ concentration, or a stochastic templating event, could create seeds that ultimately catalyze widespread plaque

deposition. Quite possibly, and as mentioned previously, the microvasculature, or the glymphatic system, may play important roles in this regard.

Why is not FAD a neurodevelopmental disease?

The mutations that result in FAD are present throughout life but nevertheless have a relatively late age of onset. The explanation for this is likely to reside in some element of brain physiology that changes with age and offsets or prevents the initial Aβ seeding event. For example, several relevant homeostatic mechanisms, such as the production of CSF (Rubenstein 1998) and proteostasis decline with age (Labbadia & Morimoto 2015), and these, and other mechanisms, could lead to a catastrophic seeding event. The same argument can be made for SAD albeit at a later age. In this model, initial Aβ seeding is a feature of the aggregatory properties of the Aβ peptide coupled to the brain's compensatory mechanisms.

Why is there no correlation between the levels of amyloid Aβ and cognitive impairment?

This issue can be partly understood from the effects of FAD mutations and *APOE4* genotype, the effects of which are to bring forward, in some cases dramatically, the age of onset of AD but not to accelerate the progression of the disease. This situation is concordant with amyloid Aβ either triggering a disease process, or surmounting some threshold before the disease is provoked (reviewed in (Karran *et al.* 2011)). In this case, one would not expect a correlation between Aβ amyloid and cognitive decline. Another way of thinking about this question is to consider that AD pathology – both amyloid and tau – can be accommodated by the brain by cell driven compensatory mechanisms until there is a deleterious cellular response that leads ultimately to system failure (De Strooper & Karran 2016).

What is the connection between amyloid and tau pathologies?

The ACH has no answer to this question currently, and in Figure 2 this is represented by 'aggregate stress'. However, there are some very intriguing trends. The discovery that mutations to *TREM2* are very significant risk factors for AD place the microglial cell centrally in the disease process (Guerreiro *et al.* 2013, Jonsson *et al.* 2013), an observation that others have made well before the genetic evidence was known (McGeer *et al.* 1988). TREM2 is a type I transmembrane protein that is expressed on microglia. A study of late onset AD patients versus controls using whole-genome gene-expression profiling (Zhang *et al.* 2013) demonstrated that a module grouping innate immunity/microglia related genes correlated best with clinical disease. *TYROBP* ranked highest as the module's potential regulator. *TYROBP*, otherwise known as *DAP12*, encodes the signalling partner for TREM2. Thus, from two orthogonal data sources the TREM2/Tyrobp signaling system has been implicated in AD. Homozygous, loss of function mutations in *TREM2* and *TYROPB* cause Nasu Hakola disease that is characterized by cystic bone lesions, white matter loss and dementia. TREM2 binds to a range of poorly defined ligands such as phospholipids, bacterial products and cell debris and receptor binding mediates microglial phagocytosis and promotes an anti-inflammatory cytokine profile (Daws *et al.* 2003, Cannon *et al.* 2012). The greatest risk for AD is associated with the R47H variant which causes loss of function by preventing

normal folding of the protein (Kleinberger *et al.* 2014). An obvious hypothesis is that TREM2 signalling plays a role in the balance of pro-inflammatory responses versus phagocytosis of Aβ plaques (Kleinberger *et al*., 2014).

A series of GWASs studies has ultimately resulted in the identification of 22 susceptibility loci (Lambert *et al*. 2013) of which a large group clearly are related to innate immune system regulation: *complement receptor 1 (CR1), clusterin, CD33,* the *MS4A6-MS4A4* cluster*, ABCA7, CD2AP, EPHA1, HLA-DRB5–DRB1, INPP5D* and *MEF2C* (Karch and Goate, 2014). It is feasible that as the effects of these genes on biological systems is unravelled, a pathway between amyloid and tau pathology will be found, and may reside in the brain's inflammatory response to amyloid deposition.

The ACH can be rejected because amyloidocentric drugs have failed

It a matter of great concern, both to adherents of the ACH and its gainsayers, that none of the amyloidocentric drugs tested to date has met their predetermined primary endpoints. However, it is one thing to test a drug and quite another to test a hypothesis. In a thorough review of the recent amyloidocentric drug discovery and development programs that have reached Phase 3 clinical testing (Table 1) it was shown that in several cases there was a significant lack of translation from preclinical to clinical science, and in only two of the programs – semagecestat and solanezumab – was target engagement measured (Karran & Hardy 2014). However, there has been no doubt that this catalogue of failure (Cummings *et al*. 2014) has been damaging: while several cogent arguments can and have been deployed to explain the lack of efficacy of these approaches (eg De Strooper, 2014), nevertheless the field may lose patience and the will to continue with amyloidocentric approaches in the absence of tangible success. The field has not been without some encouragement, however. Solanezumab was shown to have a significant effect on cognition in the Expedition and Expedition II trials in mild AD in a pre-specified secondary outcome (Siemers *et al.* 2015), and in a three year 'staggered-start' extension study following the initial blinded phase, there was evidence for a genuine disease-modifying effect (Liu-Seifert *et al.* 2015) .

Has the ACH been useful?

Undoubtedly, it has. The ACH has provided a framework that has underpinned a huge amount of both academic and industry science. While Table 1 makes for sober reading, a huge amount of data and experience has been gathered regarding trial design, patient ascertainment, drop-out rates, placebo decline, biomarkers, and clinical assessment. Significant progress has been made in field of biomarkers in particular: it is difficult to imagine that the field would have initiated the AD Neuroimaging Initiative (Weiner *et al.* 2010, Weiner *et al.* 2015) and developed a number of amyloid PET ligands in the absence of the ACH.   The field has learned from failure, although more data sharing, especially between pharmaceutical companies, would facilitate information dissemination significantly.

The field has re-defined AD in recognition that amyloid deposition occurs many years prior to the onset of symptoms, such that there is now a phase called preclinical asymptomatic amyloidosis (Sperling *et al.* 2011). This change in perspective has prompted a significant number of clinical investigations into

presymptomatic patients – a move that would have been almost unimaginable not so many years ago, but one that is nevertheless supported by our growing understanding of the disease (Reiman *et al.* 2016).

The future: are we poised for success or failure?

Table 2 gives the current status of amyloidocentric therapeutic approaches in the clinic – an impressive list. The field awaits with great anticipation the results from verubecestat and solanezumab which are the most advanced in clinical development. If either therapeutic meet their primary outcome measures, a huge amount of work, in academia and industry, would seem to have been worthwhile, and at long last AD patients and those that care for them will have been given hope. But what if they fail? The effects of FAD mutations are unequivocally to bring forward the age of disease onset: yet there are little data to support the case that they accelerate the progression of the disease once initiated (Holmes & Lovestone 2002, Godbolt *et al.* 2004, Snider *et al.* 2005, Kumar-Singh *et al*. 2006, Acosta-Baena *et al.* 2011, Karran *et al*. 2011). As FAD mutations likely all increase the probability of amyloid deposition, it could be the case that amyloid deposition triggers, but does not drive, the disease process. The long clinical silent phase between amyloid deposition and dementia suggest indeed a complicated process of cellular action and reaction, which we have called the cellular phase (De Strooper and Karran, 2016) and might lead to secondary, irreversible and damaging disturbances of normal brain homeostasis. Thus it is conceivable that amyloidocentric approaches may only provide therapeutic benefit if they are administered prophylactically in a primary prevention-type clinical trial. In the most challenging clinical scenario, and depending somewhat on the mechanism of action of the therapeutic, this would mean treating at-risk individuals prior to amyloid deposition and many years before any cognitive symptoms are manifest.   If an amyloidocentric therapy failed to act in such a trial, and presuming that it demonstrated adequate target engagement, despite all of the supporting evidence for the ACH, we would need to accept the null hypothesis. Clearly, we hope, and anticipate, that some of therapeutic approaches show efficacy well before this type of prevention study is completed. Realistically, it is unlikely that the current agents under phase 3 clinical testing will demonstrate outstanding efficacy, but to answer the question posed by the title of this review, we believe that for solanezumab at least we are poised for a modest therapeutic benefit, but a very large scientific success.

> **Commented [BDS4]:** I would not make such speculation, Basically the same holds true for the bace inhibitor trials and for the Aducanumab trial

Table 1. Outcome of Phase 3 clinical trials of amyloidocentric drugs.

Drug name	Proposed mechanism of action.	Phase 3 results
Tramiprosate Aß	Small molecule to prevent Aβ aggregation.	1052 mild to moderate AD patients randomized to 3 groups: placebo, 100, 150mg/kg bid for 78 weeks. No significant effects on primary outcome measures of ADAS-cog and CDR-sum of boxes (Aisen *et al.* 2011)
Tarenflurbil	Small molecule γ-secretase	1684 mild AD patients randomized to placebo,

	modulator to alter the ratio of Aβ peptides in favour of shorter, less aggregatory forms.	800mg bid tarenflurbil for 18 months. No significant effects on primary outcome measures of ADAS-cog and ADCS-activities of daily living (Green *et al.* 2009).
Semagecestat	Small molecule non competitive γ-secretase inhibitor to prevent production of all Aβ species.	2600 mild-to-moderate AD patients randomized to placebo, 100, 140mg semagecestat od for 76 weeks in two trials (ClinicalTrials.gov identifiers NCT00594568; NTC00762411) enrolled. Trials were halted after interim analysis showed increased incidence of skin cancer and worsening of cognition and activities of daily living (Doody *et al.* 2013).
Bapineuzumab	Humanized monoclonal antibody directed at amino acids 1-5 of Aß peptide. Mediate amyloid plaque clearance via binding to plaque and promoting microglial activation.	4500 mild-to-moderate AD patients randomized to placebo and 0.5mg/kg IV every 13 weeks for 18 months in ApoE4 carriers, and randomized to placebo and 0.5 and 1.0mg/kg IV every 13 weeks for 18 months in ApoE4 non-carriers in four trials (Clinical Trials.gov identfiers lNCT00575055; NCT00574132; NCT00676143; NCT00667810.) Trials were halted after completion of two trials demonstrated a failure to meet primary outcome measures of cognition and activities of daily living (Salloway *et al.* 2014).
Solanezumab	Humanized monoclonal antibody directed at amino acids 16-24 of Aß. Mediate amyloid plaque clearance via reduction of free concentration of Aβ peptide in the periphery and in CSF.	2000 mild-to-moderate AD patients randomized to placebo and 400mg solanezumab monthly IV for 18 months (Clinical Trials.gov identfiers NCT00905372; NCT00904683). Trials failed to meet their primary outcome measures of ADAS-cog an ADCS-activities of daily living (Doody *et al.* 2014). A secondary analysis of mild AD patients pooled from both trials showed a significant effect on cognition (Siemers *et al*. 2015). An extension study revealed that the positive effects on cognition in the mild AD group were sustained over the next two years providing evidence for a disease modifying effect (Liu-Seifert *et al*. 2015).
Gammagard® Intravenous Immunoglobulin	Mixture of human immunoglobulins that were believed to reduce peripheral Aβ levels.	Trial data currently unpublished. 390 mild-moderate AD patients randomized to 0.2g/kg/2 weeks and 0. 4g/kg/2weeks versus placebo for 18 months (ClinicalTrials.gov Identifier: NCT00818662). Gammagard failed to reach its co-

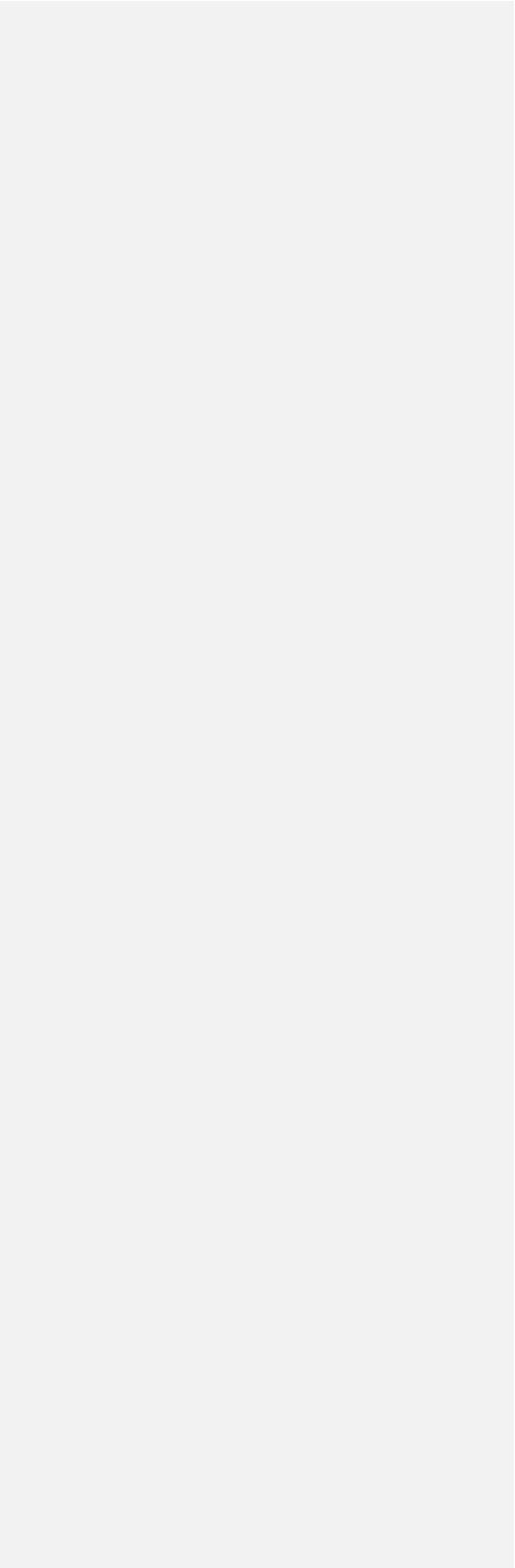|		primary outcomes of ADAS-Cog and ADCS-ADL.

Table 2. Amyloidocentric approaches in Phase 2/3 clinical efficacy testing for symptomatic AD

Name	Proposed mechanism of action.	Company	Trial characteristics
AZD3293 LY3314814.	Small molecule BACE inhibitor to prevent production of all Aβ species.	AstraZeneca Lilly	ClinicalTrials.gov Identifier: NCT02245737 Primary outcome measure: change from baseline in the Clinical Dementia Rating - Sum of Boxes (CDR-SB) Score in early AD (mild cognitive impaiment to mild AD) in a 2 year study. Aβ/amyloid relevant inclusion critiera include positive amyloid PET scan or a lumbar puncture to assay for abnormally low Aβ CSF.
Aducanumab BIIB037.	Human monoclonal antibody that binds specifically to amyloid plaque to facilitate clearance.	Biogen	ClinicalTrials.gov Identifier: NCT02477800/ NCT02484547 Primary outcome measure: change from baseline in the CDR-SB Score in early AD (mild cognitive impaiment to mild AD) in a 1.5 year study. Aβ/amyloid relevant inclusion critiera include positive amyloid PET scan.
Azeliragon PF-04494700, TTP488	Small molecule antagonist of the receptor for advanced glycation endproducts (RAGE) with multiple Aβ –related mechanims of action.	Pfizer, TransTech Pharma, Inc., vTv Therapeutics LLC	ClinicalTrials.gov Identifier: NCT02080364 Co-primary outcome measures: change from baseline in Alzheimer's Disease Assessment Scale - Cognitive (ADAS-cog); change from baseline in CDR-SB in mild AD a 1.5 year study.
Gantenerumab	Human monoclonal antibody that binds to conformational epitopes on fibrillar Aβ to facilitate clearance.	Chugai Pharmaceutical Co., Ltd., Hoffmann-La Roche	ClinicalTrials.gov Identifier: NCT0205160. Co-primary outcome measures: change from baseline in ADAS-cog; change from baseline in Alzheimer's Disease Cooperative Study-Activities of Daily Living (ADCS-ADL) scores in mild AD in a 2 year study. Aβ/amyloid relevant inclusion critiera include lumbar puncture to assay for abnormally low Aβ CSF.
Solanezumab LY2062430	Humanized monoclonal antibody directed at amino acids 16-24 of Aβ. Binds	Eli Lilly & Co.	ClinicalTrials.gov Identifier: NCT01900665 Co-primary outcome measures: change from baseline in ADAS-cog;change from baseline in ADCS-iADL scores in mild AD in a 1.5 year

	monomeric species to deplete free Aβ and reduce amyloid plaque.		study. Aβ/amyloid relevant inclusion critiera include positive amyloid PET scan or a lumbar puncture for to assay for abnormally low Aβ CSF.
Verubecestat MK-8931	Small molecule BACE inhibitor to prevent production of all Aβ species.	Merck	ClinicalTrials.gov Identifier: NCT01739348. Co-primary outcome measures: change from baseline in ADAS-cog; change from baseline in ADCS-ADL scores in mild to moderate AD in a 1.5 year study.

Figure Legends

Figure 1. Amyloid precursor protein familial Alzheimer's disease mutations.

The diagram shows the β-site amyloid precursor protein cleaving enzyme 1 (BACE1) and γ-secretase cleavage points and the protective and pathogenic amino acid changes mediated by APP mutations.

Figure 2. Modified Amyloid Cascade Hypothesis.

The scheme shows that the aggregation of Aβ is a key event but it runs in parallel with tau dysfunction. At some point, amyloid plaque provokes increased tau pathology that spreads throughout the brain. This process is accelerated by widespread deleterious effects to brain cells leading to system failure and dementia.

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