YALE JOURNAL OF BIOLOGY AND MEDICINE 89 (2016), pp.5-21.

REVIEW

YJBM

Amyloid-beta Alzheimer targets — protein processing, lipid rafts, and amyloid-beta pores

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Amyloid beta (Aβ), the hallmark of Alzheimer's Disease (AD), now appears to be deleterious in its low number aggregate form as opposed to the macroscopic Aβ fibers historically seen postmortem. While Alzheimer targets, such as the tau protein, amyloid precursor protein (APP) processing, and immune system activation continue to be investigated, the recent discovery that amyloid beta aggregates at lipid rafts and likely forms neurotoxic pores has led to a new paradigm regarding why past therapeutics may have failed and how to design the next round of compounds for clinical trials. An atomic resolution understanding of Aβ aggregates, which appear to exist in multiple conformations, is most desirable for future therapeutic development. The investigative difficulties, structures of these small Aβ aggregates, and current therapeutics are summarized in this review.

INTRODUCTION

For decades, the amyloid-beta (Aβ) and tau proteins have been the two leading targets thought to be the causative agents leading to Alzheimer's disease (AD) [1-5]. These two proteins were intensively studied because of genetic and largely phenotypic observations, namely extracellular amyloid plaques and intracellular tau tangles. The fact that these two macroscopic aggregations of protein were observable in individuals suffering from AD now appears to have led the research field slightly astray.

Just over 100 years ago, Alois Alzheimer first described the pathology and symptoms of dementia that would come to be called AD. As life expectancy continues to increase, a growing percentage of the world's population will get AD in their lifetime, with 7 percent of those ages 65 and older, and 40 percent of people 80 and older in industrialized countries being affected [6]. Despite decades of AD research, neither an effective therapy nor a vaccine has been developed. However, the wealth of molecular information now known about AD progression raises hope for therapeutic development in the next decade.

Multiple root causes of AD have been proposed that have varying levels of supporting data behind them. The amyloid theory — for decades the most widely accepted paradigm — initially posited that the extracellular amyloid plaques kill brain cells and are the causative agent leading to Alzheimer dementia. In

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†Abbreviations: Aβ, Amyloid beta; AD, Alzheimer disease; ADAM10, A Disintegrin And Metalloproteinase domain-containing protein 16; AFM, Atomic Force Microscopy; AICD, Amyloid precursor protein IntraCellular Domain; Aph-1, Anterior pharynx-defective 1; APLP1 and 2, APP like protein 1 and 2, respectively; ApoE, Apolipoprotein E; APBA, Amyloid beta (A4) Precursor protein-Binding family A (also called MINTs or X11family); APOER, apolipoprotein E receptor; APP, Amyloid Precusor Protein; BACE1, β-site APP cleaving enzyme 1 (also known as ß-secretase); BBB, blood brain barrier; ChIP-seq, Chromatin immunoprecipitation sequencing; CNS, Central Nervous System; Cryo-EM, Cryo-Electron Microscopy; CSF, cerebrospinal fluid; EFA, essential fatty acids; EGCG, epigallocatechingallate; EOFAD, Early Onset Familial Alzheimer's Disease; EPR, Electron paramagnetic resonance; FAD, Familial Alzheimer's Disease; GD3, Disialoganglioside; GD3S, GD3-synthase; GM3, Monosialodihexosylganglioside; IP3, inositol triphosphate; JIP1, JNK-interacting protein; KLC, Kinesin Light Chain; KP1, Kunitz Protease Inhibitor; LRP1, Low density lipoprotein receptor-related protein 1; mAB, monoclonal antibodies; MID, Munc 18s-interacting; MINT, Munc 18-1 Interacting proteins (also called APBs or X11 family); Nct, Nicastin; NFT, Neurofibrillary tangles; NMDA, N-methyl-D-aspartate receptor; NMR, Nuclear Magnetic Resonance; PDZ, Postsynaptic density-95/discs large/zona occludens-1 domain; PLS, partial least squares; Pen 2, Presenilin Enhancer 2; PSEN1 and PSEN2, Presenilin1 and 2, respectively; pS, picoSiemens; PTB, Phosphotyrosine binding domain; PUFA, polyunsaturated fatty acids; SRC, Sarcoma proto-oncogene; ssNMR, Solid state NMR; X11, a multidomain family of proteins containing one PTB and two PDZ domains (also called MINTs or APBs)

Keywords: Amyloid, amyloid-beta, Aß, Alzheimer, amyloid precursor protein, presenilin, secretase, plaque, dementia, pore, perforin, ß-barrel, ß-strand, dementia, lipid raft, cholesterol, ApoE, APBA, Mint, ganglioside, ß-secretase, γ-secretase.

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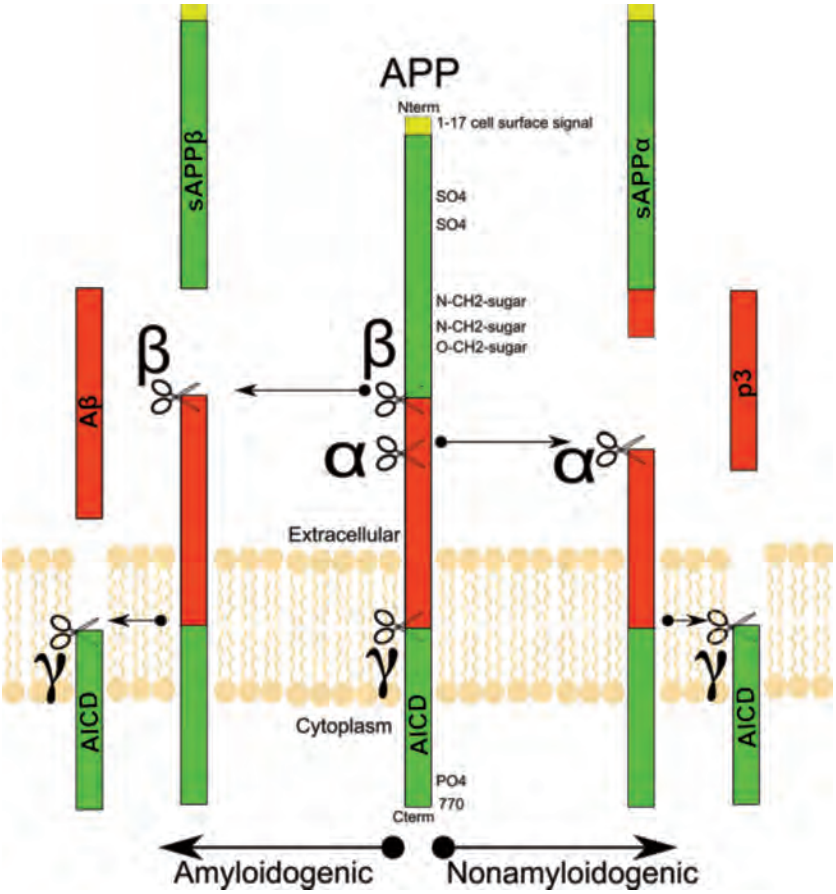
1995, genetic links leading to Early Onset Familial Alzheimer's Disease (EOFAD) were discovered [7,8].

These mutations are in the presenilin gene, a component of γ-secretase that cleaves the amyloid precursor protein (APP), releasing the amyloid peptide to the cytosol, leading to the formation of amyloid plaques (Figure 1). In mouse models, mutation of the presenilin gene causes AD-like symptoms that could be alleviated through inactivation of presenilin [9,10]. Multiple methods have proven efficacious in reducing amyloid plaques, and some have been correlated with better cognitive outcomes [11-13], whereas others have not [14-16].

All of the available treatments for AD do not augment the molecular players that lead to AD, but instead target the downstream players associated with cognitive deficits. Three of the four drugs approved by the Federal Drug Administration are cholinesterase inhibitors intended to counter the loss of cholinergic neurons observed in AD patients. The fourth drug, memantine, is an NMDA receptor antagonist that is thought to work by blocking the cytotoxic effects of excess extracellular glutamate on neurons.

Although symptomatic control by these agents has been shown to be statistically significant, their therapeutic efficacy is far from robust, and the duration of their effects is limited [17]. The low efficacy of the existing treatments necessitates the development of agents with the ability to alter or halt the progression of the disease. Ongoing drug development has focused on several aspects of AD, and aims to modify the disease.

Figure 1



Classic Amyloid Precursor Protein modification and cleavage pathway: The Amyloid Precursor Protein (APP) is cleaved by α, β, and γ-secretases. β-secretase cleavage of APP leads to amyloid plaques, whereas α-secretase results in a truncated amyloid protein, which does not aggregate.

Current drug development falls into four major categories that target:

1. The metabolic changes and insulin resistance in neurons observed with AD.
2. The inflammation associated with certain regions of the brain that are affected in AD.
3. The production of pathological forms of Tau protein.
4. The production, plaque formation, and clearance of Aβ.

The best characterized of these approaches, and the agents that have shown the greatest promise, have been those that modify Aβ levels.

While both amyloid plaques and tau tangles are macroscopically visible phenomena that correlate with AD progression, recent research suggests they may not be the causative agents of dementia [18,19]. A new model to fit available data has been put forward in which it is Aβ pre-plaque monomers or small oligomers that cause neuronal death, not the aggregated plaque [20,21]. In this model, large amyloid plaques could even inhibit neuronal death by sequestering the smaller deleterious amyloid monomers and oligomers. In this light, previous conclusions about amyloid plaque may be reinterpreted as correlative instead of causative. Promising drugs have been shown to decrease amyloid plaque formation and delay cognitive decline; however, this could be due to decreased soluble amyloid, which in turn would decrease the formation of plaques. While there is not yet consensus on the causative agent leading to AD neuronal death and dementia, inhibiting Aβ aggregation has been attempted in numerous ways as a therapeutic target, with mixed results (Table 1). The structure of Aβ aggregates, their ability to form neurotoxic pores, and the mechanism and efficacy of how current compounds target Aβ will be addressed in this review.

TAU

Neurofibrillary tangles (NFTs) comprised of hyperphosphorylated tau protein (τ), in addition to Aß plaques, are the other hallmark associated with AD. Tau interactions have been reviewed well [22-24]. Although not the subject of this review, there are interactions between τ-tangles [25] and Aβ plaques and therefore these interactions will be cursorily covered. Tau -/- neurons were protected in vitro from Aβ-induced cell death with antibodies to tau delaying motor function impairment and weight loss in multiple transgenic mouse models [23]. The link between APP metabolism and tau proteostasis is further implicated with the evidence that familial forms of AD, which exhibit mutated APP or increased levels of APP, have marked increases in intracellular tau. Recently, there has been evidence suggesting Aβ and tau interact synergistically to result in the AD phenotype of neuronal death.

Jin et al. recently isolated Aβ dimers from Alzheimer cortex and showed at sub-nanomolar concentration, without the presence of Aβ fibrils, the Aβ dimers increased hyperphosphorylated tau, followed by microtubule cytoskeleton disruption and neuritic degeneration [26]. As the amyloid theory would predict, all of these effects were modified by: knocking down endogenous tau, which rescued neuron degeneration; overexpression of human tau, which intensified the neuronal degeneration; and administration of Aβ N-terminal antibodies, which prevented cytoskeletal disruption. Furthermore, synthetic dimers had a similar, albeit reduced, effect as the natural AD dimers [27,28].

The hyperphosphorylated tau forms the hallmark aggregates termed tau tangles, but has also been shown to have other effects that are deleterious such as binding to c-Jun N-terminal kinase-interacting protein 1 (JIP1), causing it to aggregate in the cell body and impair axonal transport in AD. JiP1 normally binds the kinesin light chain (KLC) and is involved in the linkage of cargos to the kinesin-i motor complex [1,29,30].

AMYLOID PRECURSOR PROTEIN (APP)

APP can be post-translationally processed to eventually create the deleterious Aβ peptides; therefore, therapeutic targeting of this protein processing is an attractive target (Figure 1). Full-length APP also has three isoforms — APP695, APP751, and APP770 — derived from alternative splicing, which has been reviewed well by Zhang et al. [31]. In summary, the latter two, APP751 and APP770, have a Kunitz Protease Inhibitor (KPI) domain on their intracellular side [32,33] and have been found at elevated levels in the brains of those with AD [34], along with increased Aß [35].

Interestingly, APP is part of a protein family in mammals with two homologous proteins, APP like protein 1 (APLP1) and 2 (APLP2), which appear to have overlapping function as the double deletion of APLP2 and either APP or APLP1 is lethal in mice [36-38]. APP itself is a transmembrane protein that has both cytoplasmic and extracellular cleavage products that are relevant in AD. APP is initially cleaved via α-secretase or β-secretase, which leads to normal or pathological products, respectively (Figure 1).

Therefore, both α-secretase agonists and β-secretase antagonists could act as Alzheimer therapeutics. Whether α-secretase or β-secretase cleaves APP, there is intracellular release of amyloid precursor protein intracellular domain (AICD). Following α or β cleavage, γ-secretase cleavage yields extracellular p3 or Aβ, respectively. The *in vivo* role of p3 has yet to be determined. While p3 can aggregate into fibers, it is not stable in smaller oligomers, which may explain why it does not have the neurotoxic effects of Aβ [39]. Two major isoforms of Aβ exist, namely Aβ40 and Aβ42. Aβ42 is the primary amyloidogenic form in AD and by far the most studied. Therefore, shifting processing toward Aβ40 is considered protective for AD [40-42]. Also, there is a rarer Aβ43 isoform that seems to be even more detrimental than Aβ42 [43].

The non-disease function of APP and its cleavage products remains a debated topic with evidence pointing to a variety of functions including neuron growth and synaptogenesis, protein trafficking in neurons, signal transduction across the membrane [44-46], cell adhesion [47-49], and calcium metabolism [31]. Despite the current debate of what the "good" function of non-amyloidogenic APP is, there has been more clear evidence of a benefit of shifting from amyloidogenic to non-amyloidogenic pathways. The understanding of molecular players involved in shifting toward non-amyloidogenic processing of APP has resulted in actionable behavioral modifications.

For example, green tea extract contains epigallocatechin gallate (EGCG), which has been shown to increase release of non-amyloidogenic APP six-fold [50]. One way the increased EGCG does this is by binding the estrogen receptor, which increases activation of an α-secretase, namely ADAM metallopeptidase domain 10 (ADAM10) [51]. ADAM10 increases the non-amyloidogenic pathway by cleaving APP via an ERα/PI3K/Akt dependent mechanism [50]. Numerous endogenous interactions also affect APP enzymatic cleavage. APP localizes to lipid rafts and its interactions with cholesterol, Apolipoprotein E (ApoE), and other proteins in the rafts such as the Amyloid beta (A4) Precusor protein-bvinding family A (APBA) proteins, affect APP processing. All of these interactions have been investigated as therapeutics targets and are discussed below.

β-SECRETASE AND γ-SECRETASE

APP is processed into Aβ peptides by the actions of two proteolytic proteins, β-secreatse and γ-secretase. β-secretase, also known as β-site APP cleaving enzyme (BACE), is an aspartyl protease that initiates Aβ peptide

Table: Different approaches to lowering Aß levels.

Approach	Therapeutic	How it lowers Aß levels	Clinical Trial Phase completed	Clinical Efficacy
Vaccine	CAD-106	An antigen that mimics the amino acids 1-6 of Aß; produces anti-Aß antibodies	Phase II	Undetermined
Antibodies	Bapineuzumab	Binds to soluble and fibrillar forms of Aß	Phase III	Limited efficacy; discontinued because of side-effects
	Solanezumab	Binds to soluble Aß only	Phase III	Shows some efficacy in mild AD
	Crenezumab	Binds to soluble and fibrillar forms of Aß	Phase III	Shows some efficacy in mild AD
	Aducanumab	Binds to fibrillar and plaque, but not soluble forms of Aß	Phase III	No efficacy in mild to moderate AD
	Gantenerumab	Binds to fibrillar and plaque, but not soluble forms of Aß	Phase II	Undetermined, phase III trial is ongoing
ß-secretase Inhibitors	MK-8931	Blocks production of Aß by inhibiting ß-secretase enzyme	Phase II/III	Undetermined, phase III trial is ongoing
	AZD3293	Blocks production of Aß by inhibiting ß-secretase enzyme	Phase I	Undetermined, phase II/III trial is ongoing
	E2609	Blocks production of Aß by inhibiting ß-secretase enzyme	Phase I	Undetermined, phase II trial is ongoing

production [52]. While β-secretase1 is the predominant form in neural tissue, β-secretase2 isoforms are present in lower levels and therefore inhibitors of both forms could be investigated as therapeutic targets [53]. β-secretase1 in particular has been found to have increased activity in Alzheimer's patients, as well as those with mild cognitive impairment that went on to develop AD [54]. These enzymes are up-regulated in response to cellular stress such as oxidative stress, ischemia, and energy depletion [29,55]. β-secretase1 is in lipid raft domains of the cell membrane and requires glycosaminoglycans for effective cleavage [56,57].

γ-secretase is a protein complex comprised of at least four subunits: the enzymatic portion of the complex, presenilin (PSEN) 1 or 2; presenilin enhancer 2 (Pen2); nicastin (Nct); and anterior pharynx defective-1 (Aph-1) [58]. These subunits combine in a unique order to form functional γ-secretase: Nct and Aph-1 first form a dimer, followed by PSEN binding, and finally Pen2 binding, which may help in PSEN autocleavage [59,60]. More than 50 substrates (such as APP, Notch, ErbB4, and N-cadherin) can be cleaved due to recognition endowed via Nct and Aph-1 [60-62]. The fact that γ-secretase activates Notch is problematic in

that nonselective inhibitors of γ-secretase would have detrimental side effects. Notch is involved in embryogenesis, so potentially women with EOFAD could be pregnant and unable to use nonspecific γ-secretase inhibitors. Notch is also involved in neurogenesis, so even older patients could have deleterious effects due to off-target inhibition [63].

However, because increased levels of γ-secretase have been shown to lead to events associated with the AD pathogenesis [64-67], it continues to be one of the main targets for the development of therapeutics. PSENs contain six to nine transmembrane regions with both ends in the cytoplasm, and the functional γ-secretase actually cuts APP in a transmembrane region. The combined actions of β-secretase1 and γ-secretase on APP lead to the release of Aβ peptides. β-secretase1 cleaves APP to release a soluble extracellular fragment and a membrane-bound fragment, C99 [68]. C99 is then cleaved by γ-secretase at several potential transmembrane locations [69], yielding a variety of Aβ peptides ranging from 39 to 43 amino acids in length.

FAMILIAL ALZHEIMER DISEASE (FAD)

Familial Alzheimer's Disease (FAD) is an autosomal dominant condition that represents 5 percent of AD patients [70], resulting in the development of symptoms before age 65 [71]. FAD can be caused by mutations affecting three different genes APP, PSEN1, or PSEN2 on chromosomes 21, 14, and 1, respectively [72,73]. The mutations on APP all cause up-regulation of Aβ (Aβ40, Aβ42, or both) through one of three mechanisms: increased APP expression, α-secretase inhibition, or increased activity of β-secretase [71,74-76].

Aβ

It is important to note that both the amyloidogenic and non-amyloidogenic pathways are present in healthy individuals, with AD presumably being caused through increased amyloidogenic cleavage or decreased Aβ turnover [77]. In fact, healthy individuals have Aβ levels in their plasma and cerebrospinal fluid of 500pM and 3-8nM, respectively [78,79]. Clots formed in the presence of Aβ have an abnormal structure and are resistant to clearance. Aβ has been found to bind directly to fibrinogen with a dissociation constant (kd) of < 7nM, possibly modifying its tertiary structure and leading to clots [80]. The longer Aβ digestion fragments are more hydrophobic and associated with increased AD risk. Aβ 1-42 has been most extensively studied due to its abundance in patients predisposed to AD; however, Aβ 1-43 has been shown to be even more toxic in both cells and experimental animals [81]. Mutations in both APP and the PSENs can influence which Aβ peptides are formed, and missense mutations in APP and the presenilins are associated with increased release of Aβ42 and FAD [82].

Aβ STRUCTURE: WHEN, WHERE, AND WHAT

When does Aß matter — early

The hallmark of Alzheimer's historically has been the large extracellular Aß plaques that are seen postmortem. If we rewind time from the point of an AD patient's death and think about when the Aβ proteins are doing their damage, it now appears it is many years, even decades, earlier [83-85]. There is no therapeutic on the horizon that can reverse the neuronal damage associated with AD, and because it is such a protracted disease, drug development is now focused on early stages in the disease progression. For example, Eli Lilly is testing solanezumab in a Dominantly Inherited Alzheimer Network Trial on people ages 65 to 85 who are predisposed or have been diagnosed with AD, but who have yet to show clinical symptoms [86]. The recent Aβ structural insights detailed below suggest that late-stage large Aβ plaques are not the correct pharmacological target.

Where does Aß matter — lipid rafts

Historically, Aβ was measured as a single entity, not specifically by the conformation it was adopting. When measuring Aβ in aggregate, the spatial location of amyloid plaques has been found to correlate more strongly with cognitive impairment [87] than the total volume of plaque formation [88]. In particular, Aβ levels in the frontal cortex preceded cognitive decline, while Aβ levels in the entorhinal cortex correlated with measureable cognitive impairment. While levels of insoluble amyloid deposition can differentiate between disease state and control, only soluble amyloid correlated with measures of dementia [89].

Protein quantification by western blotting shows a three-fold increase in soluble Aβ of confirmed AD patients compared to controls [90]. Again, this fits with the model where amyloid fibrils are late-stage phenotypes of AD, but not necessarily causative of dementia, whereas soluble Aβ is directly deleterious and potentially the starting point of neuronal death observed in AD. PET/CT imaging has rapidly risen as a useful tool to measure Aβ *in vivo* with compounds such as monoclonal antibodies (mABs). However, PET/CT imagine can be inconsistent in certain organs, such as the kidney and the heart, and therefore origin of measurement is an important consideration when comparing to normal levels [91-94].

Currently, PET imaging modalities measure total amyloid. It remains to be seen how finely tuned compounds can detect and differentiate between different amyloid multimers. A growing area of research has shifted away from looking at Aβ in extracellular plaques [42] and instead turned toward the interaction of Aβ in the plasma membrane, namely direct interactions with lipid rafts and in β-sheet barrel pore formation [95-103]. Recent evidence has shown Aβ interactions with the plasma membrane are localized to high cholesterol lipid rafts domains where Aβ forms Ca+2 channels, thereby

disrupting membrane function. The localization to lipid rafts has led to developing therapeutics that target other proteins localized to lipid rafts, such as those involved in processing of APP.

CHOLESTEROL

The brain contains 20 percent of the body's cholesterol, of which 70 percent is found in the myelin sheaths. Unlike the rest of the body, essentially all cholesterol in the brain is unesterified [104,105]. This is important because almost all of the cholesterol in the brain is synthesized *in situ* and not transferred from the rest of the body [106]; therefore, therapeutics targeting cholesterol for AD patients will have to cross the blood brain barrier to have their desired effect. The highly polarized nature of neurons causes cholesterol trafficking to be of particular importance as has been well-reviewed elsewhere [107].

Earlier studies suggested statin use, which inhibits cholesterol synthesis, was associated with decreased AD prevalence, but later studies did not support that finding [108-110]. It has recently been shown by Liu et al. that γ-secretase cleavage of APP, forming Aβ and AICD, down-regulated low-density lipoprotein receptor-related protein 1 (LRP1) by AICD binding directly to the LRP1 promoter. LRP1, also known as apolipoprotein E receptor (APOER), regulates ApoE and cholesterol levels in the brain, and therefore could also be a target for AD treatment [111]. ApoE imports cholesterol into neurons via LRP1 and low activity of LRP1 damages neurons due to low levels of cholesterol [111]. APP knockouts increased ApoE activity and increased cholesterol levels.

The work by Liu et al. connects the two most significant genetic determinants of AD predisposition, namely APP and ApoE [112]. Cholesterol has been found to bind both Aβ and its precursor C99 via a cholesterol-binding domain (Aβ22-35) [113], with greater affinity for Aβ [113-115], suggesting another rationale for high cholesterol levels being deleterious. New therapeutics that mimic cholesterol, such as bexarotene, have been developed that can compete with cholesterol to bind Aβ and increase Aβ clearance, but do not initiate pore formation [115].

APOE

ApoE is one of the main apolipoproteins in the brain and transports both lipids and Aβ. There are three ApoE variants (ApoE-ε2, ApoE-ε3, ApoE-ε4) with ApoE-ε2 being protective against AD while ApoE-ε4 allele is the greatest risk factor. Caucasians that are homozygous for the ApoE-ε4 allele are 15 times more likely to develop AD [116]. The mechanism for ApoE's effect in AD is complex since ApoE is associated with numerous characteristics of AD, including Aβ plaque formation, τ-tangle formation, oxidative stress, inflammation, lipid homeostasis deregulation, synaptic plasticity loss and cholinergic dysfunction [117,118]. ApoE can remove Aβ plaques, with the ApoE-ε4, ApoE-ε3, and ApoE-ε2 alleles having a low, normal, and high ability to do so, respectively.

In patients with AD, ApoE has been found to localize to senile plaques (polymorphous beta-amyloid protein deposits), vascular amyloid, neurofibrillary tangles, and bind to APP [116,119-121]. Diets high in carbohydrates and low in essential fatty acids (EFA), especially ω-3 polyunsaturated fatty acids (PUFAs), increase the risk of developing AD [122]. One mechanistic hypothesis for the beneficial effects of PUFAs is that they bind ApoE, which is the primary ligand for the LDL-receptor and mediates delivery of lipids across the blood brain barrier (BBB). The relative efficacies of the ApoE-ε2 and ε4 alleles can therefore effect cholesterol homeostatis in the CNS [118]. While the BBB protects cholesterol in the brain from being sequestered by plasma ApoE; 24S-hydroxycholesterol can leave the brain crossing the BBB [123]. In fact, serum and cerebrospinal fluid (CSF) levels of 24S-hydroxycholesterol are increased in early AD, which could be due to increased cerebral cholesterol turnover during neurodegeneration [124]. While showing mixed results, there is some evidence for reducing 24S-hydroxycholesterol with statin treatment [123].

Most recently, both ε3 and ε4 isoforms of ApoE have been shown to leave the secretory pathway, enter the nucleus, and directly bind promoters and repress anti-inflammatory genes such as Sirt1 [125]. Chromatin immunoprecipitation sequencing (ChIP-seq) results suggest ApoE could interact with as many as ~1,700 gene promoter regions [125].

APBAS

The APBAs are a family of adapter proteins (APBA1, APBA2, and APBA3), previously known as MINT 1/2/3 or X11α/β/γ, which are in lipid rafts and involved in protein transport and synaptic function. APBAs have been found to bind to APP and, although mixed effects on Aβ production have been reported [126,127], are generally viewed as lowering deleterious Aβ production [128-131], which makes them possible AD therapeutic targets.

For example, APBA2 phosphorylation by SRC increases APP endocytosis leading to increased nondeleterious intracellular Aβ production, which was sequestered in vacuoles [132]. Conversely, APBA2 mutants resistant to phosphorylation increase APP entering the recycling pathway and were shuttled back to the cell surface, leading to increases in deleterious Aβ secretion [128,132]. APBAs consist of unique N-terminal regions specific for each isoform, a central phosphotyrosine binding (PTB) domain and two conserved, C-terminal postsynaptic density-95/discs large/zona occludens-1 (PDZ) domains [133]. The PTB domain interacts with a conserved NPTY (Asn-ProThr-Tyr) cytoplasmic motif of the APP that participates in APP trafficking and processing [134]. APBA1 and

APBA2 interact with other proteins as well, including Pen1presenilin-1, which is required for γ-secretase activity, through the PDZ domain [135]. Surprisingly, transgenic mice that overproduce Aβ peptides were found to decrease amyloid plaque production when crossed with APBA knock-out mice [126].

Additional studies have shown that the N-terminus of APBA contains a Munc18s-interacting (MID) and Munc18a domain, that bind and retain APP, and can suppress Aβ secretion [136]. The role of APBAs with APP remains complex with APBAs being reported to increase β-secretase activity [126] and decrease γ-secretase activity [137], which are the first and second cleavage steps, respectively, in the amyloidogenic processing of APP to Aβ (Figure 1). Taken together, these studies suggest that regulation of APP processing by APBAs is achieved by interactions with both Pen1presenilin-1 and APP.

GANGLIOSIDES

Another enriched component of lipid rafts is gangliosides, a form of glycosphingolipid containing sialic acids on the sugar chain. Exogenous gangliosides have been found to elicit multiple neurotrophic effects [138] and not only help lipid rafts form, but stimulate Aβ production, directly bind Aβ, and also increase amyloid plaques [139-142]. GD3-synthase (GD3S) converts a-series ganglioside (GM3) to b-series ganglioside (GD3) via addition of a sialic acid and is thereby the branch point for a- and b-series gangliosides. This controls the level of a major b-series ganglioside in the brain, GM1, which greatly increases Aβ production [143].

A negative regulatory feedback loop has recently been elucidated by Grimm et al. in which GD3 increases APP cleavage forming Aβ and AICD, which work via two mechanisms to synergistically block production of GD3 [139]. Aβ directly binds GM3, reducing the GD3S reactant pool while increasing the ratio of GM3:GD3, which is an increase in the overall cell ratio of a-series:b-series gangliosides. AICD lowers GD3S at a transcriptional level (via binding the adaptor protein F365) [139]. It is worth noting that since Aβ is secreted, taken up by cells [144,145], and present in large concentrations in the cytosol of neurons [40,146,147], Aβ-GM3 binding could take place along the secretory pathway, in endosomes, by the cell lipid bilayer, as well as extracellularly [31]. In addition, while GM3 overall lowers Aβ production, one of its downstream a-series gangliosides, GM1, has been shown to have mixed effects by increasing Aβ generation and aggregation in vitro [148], while potentially being neuroprotective *in vivo* [149-151].

WHAT CONFORMATION OF Aβ MATTERS

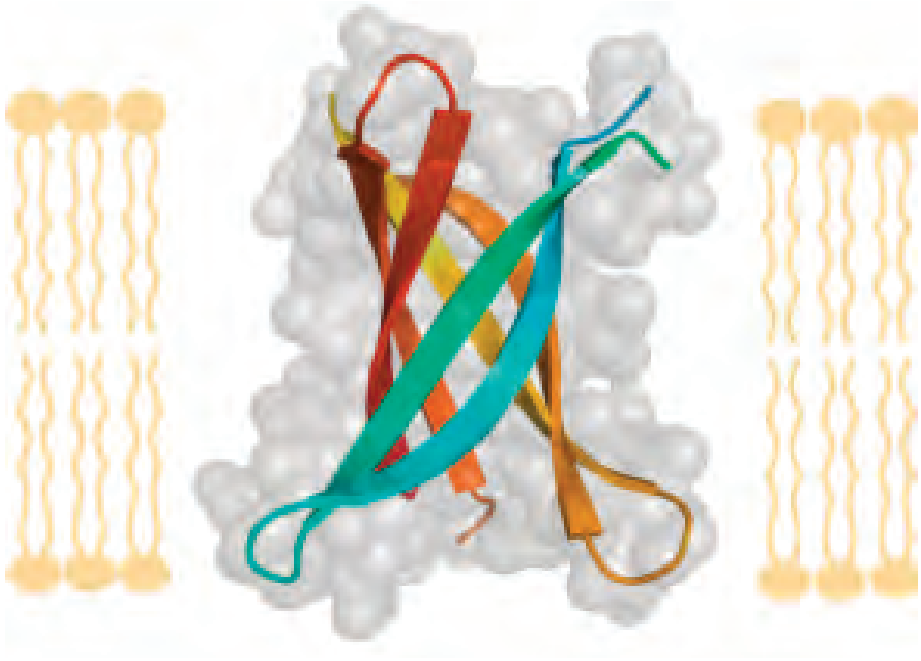
The investigation of Aβ aggregates has been greatly stymied by their lack of rigidity. The Aβ fibrils do not take on a single conformation, so there is not a single X-ray crystal structure that represents the only targetable conformation. For example, the Asp23-to-Asn mutant of Aβ (D23N-Aβ1–40), which is associated with EOFAD, can contain either parallel or antiparallel β-sheets [152,153]. Each Aβ peptide can form a β-sheet and two Aβ peptides can interact forming a β-sheet dimer, which has been termed a steric zipper. This dimer interaction can be classified structurally based on three variables: arrangement of the monomer β-strand internally (parallel or antiparallel), orientation of β-strands (same edge up for both sheets, or one up and one down), and orientation of the faces of the β-sheets (face-to-face [most common], or face-to-back). All eight of these combined possibilities have been observed experimentally with short peptides [154,155]. Initial investigations suggested the antiparallel fibrils are less stable and propagate less efficiently, but may nucleate more efficiently than parallel fibrils. Both antiparallel and parallel fibrils were found to be cytotoxic [156]. It should therefore not be surprising that several models for amyloid structure have been put forward [157].

For decades, the field had followed large Aβ and tau aggregates, which it continues to do. Indeed the aggregates are correlated with disease progress, with tau aggregates being especially positively correlated with worsening dementia. It has recently been shown that Aβ aggregates from two to 12 subunits may be the deleterious form [114]. In this paradigm, the large amyloids plaques seen postmortem historically could actually act as "sinks" to sequester the deleterious Aβ monomers. If the Aβ sequestering benefit is validated, it could lead to changes in therapeutic avenues. For example, breaking up amyloid plaques in an Alzheimer's patient could be beneficial. This should only be the case if broken up into 13mers or larger since 3-12mers seem to form toxic pores. A single amyloid plaque 10,000 Aβ units long has at most two sequestering ends; if broken up into 100 Aβ units, it would have 100 times the sequestering power. In line with this concept, compounds have recently been discovered that increase Aβ aggregation, yet save cells, suggesting the Aβ plaques sequestered the more deleterious soluble Aβ multimer [18,19].

Aβ AGGREGATION

Amyloid plaques created in the lab form multiple structures dependent on the conditions set. An interesting parallel can be made to the field of crystallography, where scientists search for just the right buffers to force normally soluble proteins to form crystal lattices. In contrast, the amyloid protein is so prone to oligomerization that discerning the meaningful *in vivo* structure from the multitude of in vitro structures has been difficult. A plethora of structural methods have been used to investigate the dynamic Aβ aggregation including solution [158] and solid state nuclear magnetic resonance (NMR) [159-161], electron paramagnetic resonance (EPR) [162], cryo-electron microscopy (cryo-EM) [163,164], atomic force microscopy (AFM)

Figure 2A: Amyloid ß-barrel



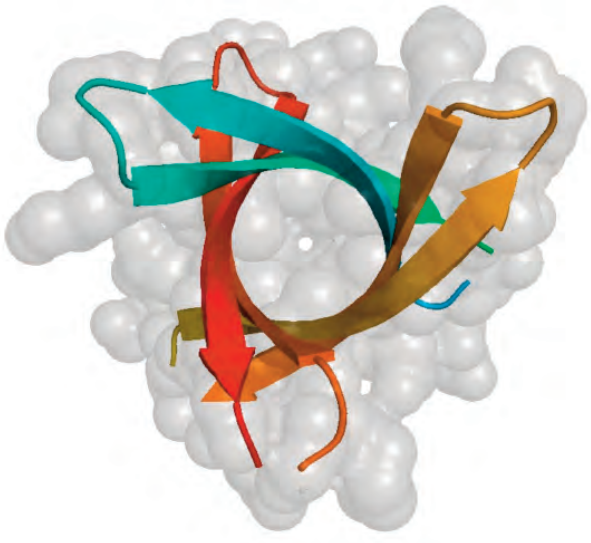
2A is a cross-section view of the membrane.

2A,2B: A six-strand ß-barrel crystal structure (pdb entry=3SGR), shown as a membrane cross-section and rotated 90 degrees looking though the pore, made of three ß-sheets. Colored by strand, each strand has a tighter ß-sheet partner on one side (teal with cyan, yellow with light orange, red with dark orange) and a weaker ß-sheet partner on the other side (teal with cyan, yellow with light orange, red with dark orange).

[165-167], X-ray [155,168,169] and computational molecular dynamics [170-174] with implicit and explicit water. Investigating these in vitro amyloid aggregates is helpful in creating therapeutics.

For example, Agopian et al. [175] formed two significant amyloid formations of Aβ40, creating an untwisted conformation if agitated or twisted if not agitated. Both NMR [176] and EPR [177] work have shown that Aβ fibrils form a parallel in-register network in which the same residues from each monomer pack against each other. It is worth noting that only a couple of years ago, Liu et al. described, for the first time, an out-of-register amyloid fibril in which the full complement of hydrogen bonds between monomers was not formed [178]. Atomic resolution of the deleterious Aβ oligomer hydrogen bonding network is vital for rational development of compounds, which will disrupt this interaction. Since backbone hydrogen bonding of β-sheets always favors planar structure [179], understanding packing forces that may cause twists in fibrils is important. Using EPR spectroscopy and 14 spin-labeled positions, it was found that agitated (untwisted) fibrils had stronger inter-strand interactions. The two hydrophobic regions stretching from residues 17-20 and 31-36 had the strongest interactions with the inter-strand distance estimated to be 0.2Å closer in the untwisted compared to the twisted fibril [175].

Figure 2B: Amyloid ß-barrel



2B looks down the barrel of protein pore and through the membrane.

Aβ PORE FORMATION CAUSING MEMBRANE CA+2 CONDUCTANCE

One of the most exciting amyloid discoveries, now two decades old, may be Aβ's ability to form pores in neurons. Using electrophysiological techniques, Arispe et al. showed amyloid aggregates form channels in planar lipid bilayers [180,181]. While it has clearly been shown that Aβ at millimolar concentrations can cause ion conduction across the cell membrane [182-186] with a conductivity of 100pS/pore [187], the exact structural mechanism is debated. Multiple studies have shown Aβ allows Ca+2 influx, which can be blocked by Zn+2 ions [188-190].

Figure 2C

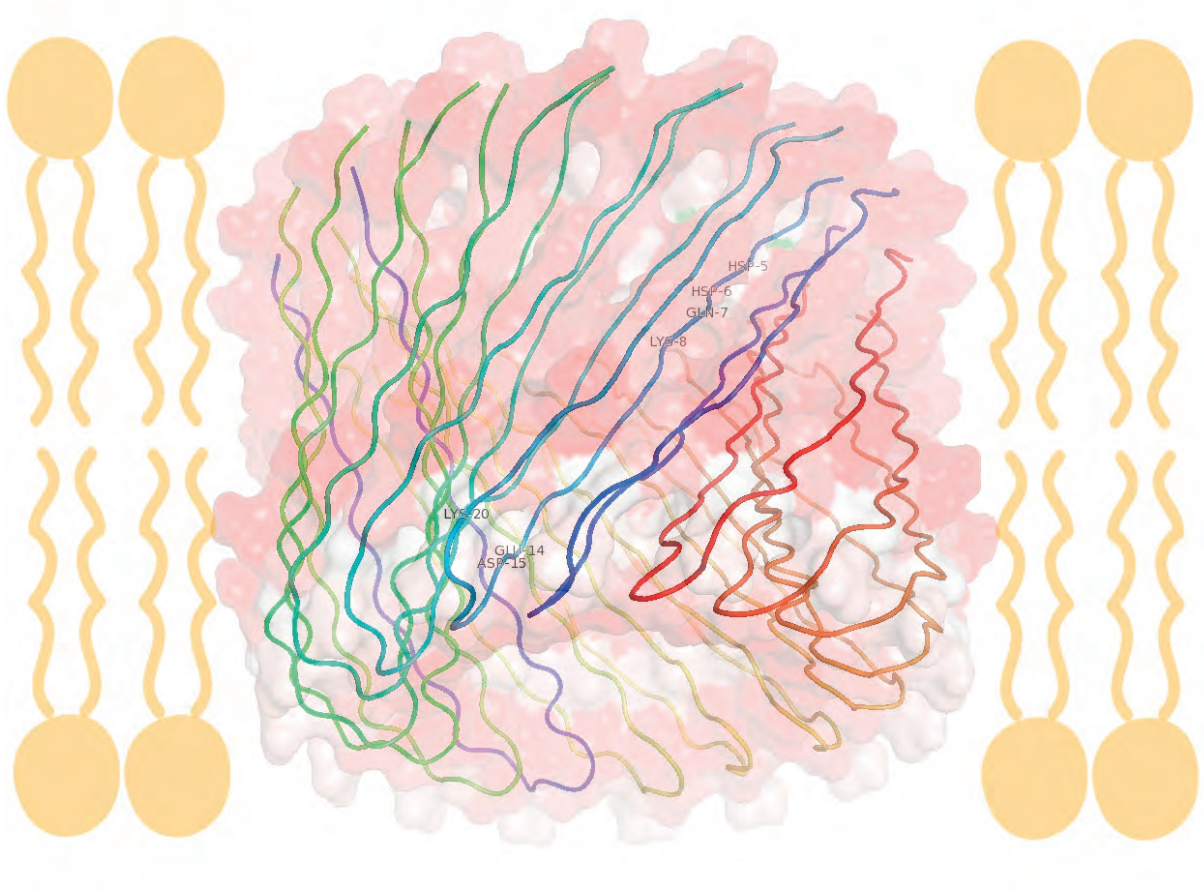
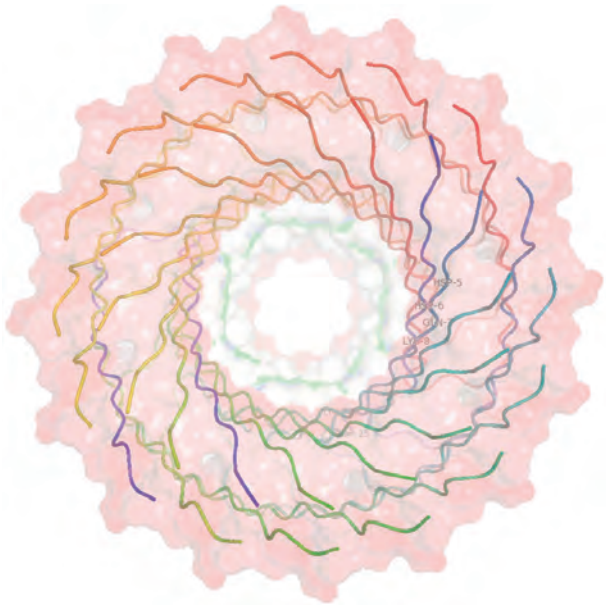


Figure 2D



2C,2D: A 16 ß-strand ß-barrel, shown as a membrane cross-section and rotated 90 degrees looking though the pore, composed of eight Aß subunits, computational modeled by dynamics to match ssNMR data. The molecular surface is color coded by hydrophobicity (red = nonpolar, white = polar, green = cysteine). The images were made using pymol [231].

Disruption of calcium channels can cause multisystem disorders collectively, termed "calciumopathies."

In the brain, Ca+2 acts as a second messenger to regulate membrane potential, excitability [191-193], synaptic plasticity, and memory encoding [194-197]. Calcium release from the ER, which has Ca+2 levels multiple orders of magnitude greater than the cytosol, is triggered by both ryanodine and inositol triphosphate (IP3) receptors, which are activated by cytosolic calcium and IP3, respectively [198-200]. Increased Ca+2 influx into the cytosol via an Aβ pore leads to increased ER calcium release, activating ER and mitochondrial stress responses, which leads to apoptosis [201-205].

Interestingly, Aβ oligomers administered intracellularly have also been shown to activate ER Ca+2 release by increasing IP3 even when in a Ca+2 free solution, hence not related to pore formation [198]. The mechanism of IP3 stimulation is still being investigated. Several mechanisms have been presented for this membrane Ca+2 permeability [100], including tension induced poration [206,207] and disassembly of the lipid bilayer by both oligomers [96,100], as well as the interaction of the larger

fibrils with the membrane [208,209]. Aβ pores have been measured with various numbers of subunits (3-6 Aβ monomers) by AFM. Most of the pores (two-thirds) are tetramers or pentamers, with one-third being trimers or hexamers [210]. Prangkio et al. developed controlled aggregation conditions and used partial least squares (PLS) to show that oligomers in the 4-13 range promoted both pore formation and cytotoxicity, while monomers, dimers, and trimers were negatively correlated with both of these deleterious outcomes.

Models of six-stranded β-barrels formed by the last third of Aβ42 show hydrophobic residues and a glycine in the core, which is shielded from bulk water by the central and N-terminus regions, which can adopt both sheet and helix conformations (Figure-2A-B) [211]. Shafrir et al. predicted how these antiparallel six-stranded β-barrels interact to form larger fibrils as well without seeing any large energetic penalties for the transition (animations of this transition were made available as well) [211]. While the Aβ β-barrel structure is most easily investigated in silico, due to its dynamic and membrane bound structure, the Aβ β-barrel has been investigated at the atomic level by X-ray [157] and NMR [212].

In 2012, a β-barrel compatible with a sequence segment from Aβ protein was crystallized by Eisenberg et al. at 1.4Å resolution [157]. The structure could not be solved by molecular replacement with fiber-like structures and after bromine substitutions, the novel X-ray structure proved to be a six-stranded antiparallel β-barrel termed cylindrin. Each strand in cylidrin has a strong interface on one side made from 12 hydrogen bonds and a weak interface on the other side made by eight hydrogen bonds (Figure-2A-B).

Computational simulations have been used to suggest how Aβ pore formation originates. Jang et al. used explicit solvent molecular dynamics simulations to model 12-, 16-, and 20-mer Aβ barrels and had results that were consistent with solids state NMR (ssNMR). For example, the 16mer barrel modeled had an outer diameter and pore diameter of ~7.2nm and ~1.6nm, respectively (Fig-2C-D) [212]. It should be noted these measurements are similar to the 1-2nm pore size measured by ssNMR, which is physically wide enough for water to flow through. Unlike the cylindrin crystal structure, all β-sheet structures were parallel, meaning the pore had all β-loops on one side and all N- and C-terminal ends on the other side.

Two aspects to note are the pore size opening on each side and the channels hydrophobicity. The side of the pore with all β-turns has a smaller opening and a patch of residues (Glu14, Asp15, and Lys20) from each Aβ peptide, which forms a hydrophilic ring. On the opposite side of the pore, made of the N- and C-terminal ends, the residues Glu7 and Lys8 form a hydrophilic ring (shown as white in Fig2D). The peptide chain in these structures would be termed "coil" in a normal protein, but are in a pre-β-sheet layout and represent a possible early stage of pore formation (Fig-2C-D) [212].

Regular antiparallel β-barrels come in two flavors as defined by their shear number (S) with a higher number indicating greater displacement between the edges of β-sheets, slope of strands in degrees (D) relative to the central axis of the barrel, and the number of hydrogen bonds (H) on each side of the β-strands, which may alternate. Normal β-barrels have either [S=12,D=56º,H=10,8] or [S=6,D=37º,H=10,10]. Cylindrin shares aspects from both of these groups with a topology [S=6,D=35º,H=12,8] [157]. While Aβ pores have been modeled with multiple sheets, and even with α-helical structures [113], the six-strand model X-ray model is useful for binding, folding, and modeling purposes.

CURRENT THERAPEUTIC ATTEMPTS, SUCCESSES, AND FAILURES

Attempts to decrease Aβ levels have been attempted for over two decades, however even when effective, the side effects have proven too deleterious compared to little or no cognitive benefit. The first attempt at modifying Aβ levels was performed using immunization with a synthetic full-length peptide. Although this approach had shown robust effects in animal studies, and lowered total Aβ levels in the brains of human subjects, an accompanying T-lymphocyte mediated meningoencephalitis halted further development [213,214]. Current vaccination approaches have reduced T-lymphocyte activation by using an attenuated form of Aβ (Aβ-1-6) [215]. The development of many Aβ vaccines were terminated after phase II clinical trials because they showed low clinical efficacy. CAD106, which consists of multiple copies of Aβ1-6, has been shown to consistently increase Aβ-antibody titer, without inducing T-lymphocyte activation [216]. The safety profile of CAD106 suggests that it could be a potential therapeutic option for AD, but its clinical efficacy has yet to be determined.

The leading approach in developing agents that lower Aβ levels is passive immunity. Animal studies, in which systemic administration of anti-Aβ antibody was shown to cross the blood brain barrier and induce an immune response to Aβ were the first studies to show the effectiveness of passive immunity [217]. These initial studies lead to the development of an humanized anti-Aβ antibody, bapineuzumab [218]. Bapineuzumab binds to the soluble and fibrillar forms of Aβ and is well-tolerated at doses less than 2mg/Kg [219]. In phase III studies of mild to moderate dementia associated with AD, bapineuzumab was shown to decrease total brain Aβ load, compared with placebo control, using positron-emission tomography, but did not significantly improve cognitive or functional outcomes as measured by the Alzheimer's Disease Assessment Scale and Disability Assessment for Dementia [220].

A number of other anti-Aβ antibodies have been developed that target different regions and forms of Aβ (Table 1) [221]. Unlike bapineuzumab, solanezumab is a monoclonal antibody that recognizes the soluble

monomeric, but not the fibrillar, form of Aβ. By interacting with the soluble form of Aβ, solanezumab is thought to block the more neurotoxic form of Aβ [222]. In a multicenter phase III trial with 2,052 patients with mild to moderate AD, no improvement in cognition or functional ability was observed in subjects receiving 400mg of solanezumab every four weeks for 18 months when compared with the placebo group [223]. However, a subgroup analysis showed a reduction in decline of cognition in the group with mild AD [221]. Similarly, crenezumab, an anti-Aβ antibody that binds to both the soluble and fibrillar forms of Aβ, also seems to be efficacious, although not robust, in patients with mild AD, but not in those with moderate AD [224]. The clinical effectiveness of these agents in the early stages of AD, despite the reduction in Aβ levels, suggests that the effectiveness of disease-modifying agents may depend on when they are employed. An effective AD treatment will likely need to use strategies to identify early stages of the disease, potentially before symptoms of the disease begin to manifest, in addition to the disease-modifying agent.

Blocking the production of Aβ, by modifying the processing of APP, is another strategy for lowering Aβ levels. The target of this approach has been the enzyme β-secretase. Initially, most of the inhibitors were peptide-based, but ongoing work has focused on small molecule inhibitors [225]. Development of β-secretase inhibitors is in its early stage, and to date, only two drugs (MK-8931 and AZD3293) that inhibit β-secretase activity, and thus reduce the production of Aβ, have reached phase III clinical trials. MK-8931 and AZD3293 have been shown to be effective in lowering Aβ levels and are well tolerated [225,226]. The clinical efficacy of β-secretase inhibitors is still being determined, but like antibodies that lower Aβ, β-secretase inhibitors are likely to be more effective in the early stages of AD.

CONCLUSIONS AND OUTLOOK

The dynamic and numerous binding interactions of the Aβ peptide has slowed Alzheimer's research for decades. While there is still not a single accepted causative, structurally characterized, and targetable Aβ protein structure, there are a number of leading candidates being pursued. The Aβ pore formed by six (or more) β-strands has been computationally predicted by multiple groups and fits experimental evidence, such as AFM images. Compounds that prevent the pore formation, followed by Ca+2 influx into the cytosol, followed by Ca+2 efflux from the ER into the cytosol, could halt neuronal death and associated cognitive decline. Likewise, new targets that disrupt lipid rafts, where Aβ localizes, could delay onset of disease (as has already been shown with increased ω-3 fatty acids [227-230]). Following the effect of new therapeutics on lipid raft formation and calcium flux should provide finer resolution of cellular progression during Alzheimer's at the molecular level.

Unfortunately, despite all of this recent Aβ structural elucidation suggesting new avenues for targets, it needs to be noted that many historically failed therapeutics should have proven effective if the Aβ pore were the causative agent. The monoclonal antibodies to Aβ (e.g. solanezumab, bapineuzumab, and crenezumab) could have been detecting the wrong conformation of Aβ, namely monomers or fibrils. However, both β–secretase and γ–secretase drugs designed to lower the total amount of Aβ still seem like they should have proven effective. Developing compounds that can differentiate and detect the difference between multimers of Aβ will likely prove very difficult. Such compounds could inherently require large surface contacts to be able to differentiate. For example, if tetramers and octomers had the same repeating structure and only differed in their size, a compound to recognize the octomer as different would have to be at least a little bit longer than the tetramer to detect the higher multimer of Aβ.

The Alzheimer's research field is therefore again left in a situation it has become all too familiar with: learning more about the disease without an overwhelming obvious best path forward.

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