CURRENT STATE OF ALZHEIMER'S DISEASE RESEARCH AND THERAPEUTICS

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Hsp90 Modulation for the Treatment of Alzheimer's Disease

Abstract

Hsp90 serves as the master regulator of the prosurvival, heat shock response. Upon exposure to cellular stress or small molecule inhibitors of Hsp90, various heat shock proteins are induced to assist in the rematuration of misfolded proteins. Several neurodegenerative diseases, including Alzheimer's disease, manifest through the accumulation of misfolded proteins, suggesting that induction of the heat shock response may provide a viable approach toward the management of such diseases. In this chapter, the rationale for such an approach and potential therapeutics are discussed.

I. Introduction

Nearly all age-dependent neurodegenerative diseases are characterized by the accumulation of misfolded proteins that form distinctive types of aggregates within or outside the brain or spinal cord neurons and glia, a process often called "proteotoxicity." Although the presence of these neuropathological lesions was used for many years to establish a definitive diagnosis for diseases such as Alzheimer's disease, it has only been within the past three decades that the actual proteins found in the brain lesions have been identified. We now know that the "senile plaques" of Alzheimer's disease (AD) are primarily composed of aggregates of a 40–42-amino acid peptide (Aβ) derived from the large amyloid precursor

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protein (APP) by sequential proteolysis (Glenner & Wong, 1984). Neurofibrillary tangles (NFT), the second lesion characteristic of AD neuropathology, are composed of fibrils of hyper-phosphorylated Tau—a microtubule-associated protein (Grundke-Iqbal et al., 1986). Parkinson's disease, the second most common progressive neurodegenerative disorder, leads to the development of Lewy bodies composed primarily of fibrillar α-synuclein (Spillantini & Goedert, 1998). The identities of aggregated proteins in some less frequent, but equally debilitating nervous system diseases are also known. These include diseases such as Huntington disease with aggregates of the polyglutamine-rich protein huntingtin (DiFiglia et al., 1997), amyotrophic lateral sclerosis (ALS) with inclusions of superoxide dismutase1 (SOD1) (Bruijn et al., 1998) and the RNA/DNA binding protein TDP-43 (Arai et al., 2006; Neumann et al., 2006), the spongiform encephalopathies with prion protein aggregates (Bolton et al., 1982), and the "Tauopathies" with fibrillar aggregates of mutated Tau (Lee et al., 2001). Proteins associated with these neurodegenerative diseases do not share obvious sequence or structural homology and, in fact, appear in different cell types and in different regions of the central nervous system (CNS). It is this diversity that gives rise to the early clinical picture that emerges in patients with these diseases. Nevertheless, these conformational diseases do have some characteristics in common.

Current research on the properties manifested by proteins found in the conformational diseases has revealed that most are monomers that undergo conversion from α-helical structures to misfolded β-sheet-containing proteins that are strongly prone to self-aggregation and become pathogenic. It appears that the proteins initially form oligomers that act as seeds to promote further misfolding by serving as templates to catalyze the growth of polymers. As the nucleation process progresses, the polymers become insoluble and are eventually deposited in the brain tissue, forming plaques, tangles, Lewy bodies, and other inclusions characteristic of specific neurodegenerative diseases (Soto & Estrada, 2008). However, much evidence now supports the hypothesis that, at least for Aβ, the intermediates or soluble oligomers are the toxic species that actually lead to synaptic dysfunction and challenge neuronal viability (Walsh & Selkoe, 2007). The deposits may result from the failure to control aggregation or to sequester insoluble assemblies outside the neurons. Therefore, maintaining solubility or facilitating the disposal of such oligomers is the challenge faced by the cells' protein quality machinery. Not surprisingly, many drug discovery efforts are now underway to design strategies for optimizing the functions of that cellular machinery.

Perhaps the most important characteristic shared by the protein conformational diseases is their association with the process of aging. The major risk factor associated with the emergence of clinical signs and

symptoms of diseases such as AD is increased age. It is true that mutations in the genes for several of the misfolded proteins lead to familial forms of the diseases with an earlier onset, but the vast majority of cases are sporadic and emerge late in life. So what is it about the aging process that makes the brain vulnerable to "proteotoxicity?" Certainly no clear answer exists at this time, but the question has sent scientists on a quest to understand the systems that cells use to maintain protein quality control across the lifespan, namely the systems that fold nascent proteins, monitor the state of extant proteins, and refold or induce degradation of those that are misfolded. These investigative efforts have led to a wealth of new information about the vast network of the "molecular chaperones" and the pathways through which they enable cell protection against proteotoxic stresses (Kopito & Ron, 2000; Powers et al., 2009). The molecular chaperones, many of which are called "heat shock proteins (Hsps)," are ubiquitous and highly conserved proteins at the center of conformational homeostasis, and substantial evidence indicates that these systems become less efficient with age, possibly due to enhanced oxidative stress, which leads to oxidation and nitration of proteins, including the chaperones themselves. Such conditions could easily overload the system and allow for the accumulation of more misfolded proteins (Cuervo & Dice, 2000; Lund et al., 2002; Tonoki et al., 2009). Enhancing the protein quality control capacity by elevating chaperone protein expression is one approach toward halting or reversing the deterioration process associated with aging. Since Hsp expression is tightly regulated by heat shock factor 1 (HSF-1), the discovery of new molecules that induce expression is likely to provide agents that can protect the brain against devastating neurodegenerative cascades. Extensive research efforts including genetic and high-throughput screening approaches have identified a handful of genetic and chemical activators of HSF-1 (Calamini et al., 2012; Neef et al., 2010; Santagata et al., 2012; Silva et al., 2011,). Although indirect activation of HSF-1 by modulating posttranslational modifications such as phosphorylation, sumoylation, acetylation, direct activation of HSF-1 by interfering with protein–protein interactions, or the promotion of HSF-1 trimerization have been proposed (Neef et al., 2011), pharmacologically activating HSF-1 by suppressing the proteins that negatively regulate HSF-1 function is the most well-characterized approach. Since HSF-1 activation is tightly regulated by heat shock protein 90 (Hsp90), one promising strategy is the development of small molecules that modulate Hsp90, which acts in concert with other chaperones, transcription factors, kinases, binding partners, and substrates to maintain cellular "proteostasis" (http://www.picard.ch/downloads/Hsp90facts.pdf). This review is focused on efforts to develop potential therapeutic agents that target the Hsp90 protein folding machinery as a novel approach toward the treatment of AD and related neurodegenerative diseases.

II. Hsp90 Complexes in Alzheimer's Disease

Hsps represent a large family of molecular chaperones that are highly conserved across a wide array of organisms, ranging from bacteria to homosapiens (Blagg & Kerr, 2006; Richter & Buchner, 2006). As a cell-protective mechanism, Hsps are capable of modulating the proper folding of nascent polypeptides, assisting the refolding of denatured proteins, and directing damaged proteins to the ubiquitin-proteasome pathway for degradation. Together, these processes maintain the cell protein homeostasis under normal conditions and protect the cell from intrinsic or extrinsic insults that may arise upon cellular stress (Taipale et al., 2010). Hsp90 is the focal point of the chaperone system and is responsible for organizing the heteroprotein complex that is in charge of protein folding or degradation. In fact, Hsp90 is the most abundant molecular chaperone in the cell, and accounts for ~1–2% of total protein in normal cells. The Hsp90 chaperone family consists of four isoforms: the inducible Hsp90α and the constitutively active Hsp90β in the cytosol, the 94kDa glucose-regulated protein (Grp94) in endoplasmic reticulum, and Hsp75/tumor necrosis factor receptor associated protein 1 (TRAP-1) in the mitochondrial matrix (Blagg & Kerr, 2006; Krukenberg et al., 2011). In humans, Hsp90 exists as a homodimer, wherein each monomer contains three highly conserved domains (Fig. 1): a 25kDa N-terminus that includes an ATP-binding pocket, a 35kDa middle domain that is used for substrate recognition, and a 12kDa C-terminus that contains an MEEVD motif for the binding of cochaperones that express tetratricopeptide repeats (TPR-domain) (Bracher & Hartl, 2006; Krukenberg et al., 2011).

Hsp90 functions by forming a multicomponent complex with cochaperones including Hsp40, Hsp70, Hop (Hsp70 and Hsp90 organizing protein), Cdc37, and p23 that serve to recognize client proteins and assist their binding to the Hsp90 heteroprotein complex (Dickey et al., 2007; Waza et al., 2005). CHIP (carboxy terminus of Hsp70-interacting protein) is another important protein quality controller in this chaperone system. As a

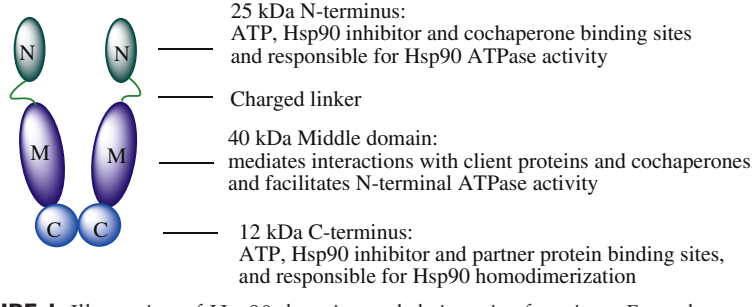

FIGURE 1 Illustration of Hsp90 domains and their major functions. For color version of this figure, the reader is referred to the online version of this book.

cochaperone, CHIP binds to Hsp70 through the TPR domain and exhibits intrinsic E3 ubiquitin ligase activity that promotes ligation and chain elongation of substrates, and subsequently directs substrates to the ubiquitin-proteasomal system (UPS) for degradation (Ballinger et al., 1999; Jiang et al., 2001; Petrucelli et al., 2004). Consequently, CHIP functions as a bridge that links chaperone function to the UPS and modulates the cellular balance between protein folding and degradation (Connell et al., 2001; McClellan & Frydman, 2001; Meacham et al., 2001). Although the exact mechanism of how the Hsp90 protein folding machinery regulates the cellular balance between folding and degradation is still not fully understood, it is postulated that Hsp40 and Hsp70 recognize aberrant or misfolded client proteins by binding to their exposed hydrophobic amino acids to prevent aggregation (Fig. 2). Through the assistance of Hop, the client protein is passed from Hsp40/Hsp70 to Hsp90. The client protein can then be refolded upon the binding of cochaperone p23, which completes the maturation cycle by releasing the folded protein to regenerate the chaperone. However, if the client protein is damaged or unable to undergo conformational maturation, it is passed to CHIP, which binds both Hsp70 and Hsp90, and begins ubiquitin ligation. Alternatively, a client bound to Hsp70 that is damaged can undergo direct degradation through the UPS by recruiting CHIP without the assistance of Hsp90 (Adachi et al., 2009; Dickey et al., 2007; McClellan & Frydman, 2001). In the former scenario, Hsp90 is also degraded as CHIP binding and appears to prevent client protein release (Dickey et al., 2007). Hsp90 tightly regulates the activity of HSF-1—a master regulator of the heat shock response. HSF-1 binds Hsp90 under normal conditions; however, in the presence of stress, it dissociates from Hsp90. Once released, HSF-1 is phosphorylated and subsequently trimerizes before entering the nucleus to bind elements that regulate the

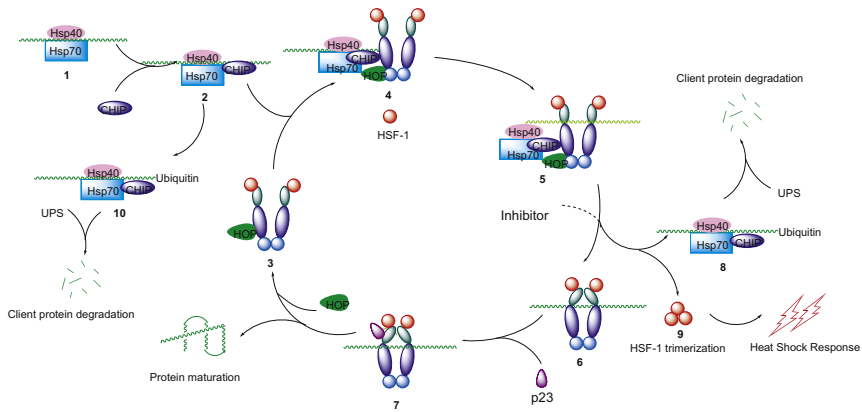

FIGURE 2 Possible Hsp90 client protein refolding/degradation pathways. For color version of this figure, the reader is referred to the online version of this book.

heat shock response. The heat shock proteins that are induced in response to HSF-1 transcriptional activation include Hsp27, Hsp40, Hsp70, and Hsp90. The expression of these chaperones expands the buffering capacity of the cell and restores protein homeostasis under stressful conditions (Dickey et al., 2007).

Unfortunately, in AD and other age-related neurodegenerative diseases, this protective mechanism appears limited and/or the aberrant proteins have accumulated beyond the buffering capacity of the heat shock response (Adachi et al., 2009; Morimoto, 2008; Shamovsky & Gershon, 2004). Consequently, the balance between protein production and clearance is no longer at equilibrium, and the accumulation and aggregation of toxic protein species such as amyloid-β occur (Hardy & Higgins, 1992). As proposed in tauopathy, this long-term and progressive insult to neuronal cells may cause abnormal downstream kinase activities that promote tau hyperphosphorylation and aggregation that leads to the deposition of neurofibrillary tangles in the brain, another characteristic feature of AD (Morris et al., 2011; Salminen et al., 2011). Additionally, tau hyperphosphorylation results in decreased binding affinity for microtubules and results in microtubule destabilization and axon damage (Lau et al., 2002). Although aberrant tau accumulation and aggregation can be triggered by chronic accumulation of β-amyloid, it appears that eventually it becomes independent of the initial trigger, and ultimately may be self-reinforcing (Golde et al., 2011). This latter scenario may partially explain the failure of β-amyloid-directed therapies in the clinic due to the manifestation of multiple AD symptoms. Moreover, it has been proposed that amyloid-β accumulation suppresses the expression of CHIP and subsequently alters the mechanism by which proteins are cleared through the UPS (Oddo et al., 2008). It appears as though AD and possibly other neurodegenerative diseases may result from an inefficient or insufficient heat shock response or a related UPS impairment, which may represent the initial trigger for disease onset.

Accordingly, restoration of an impaired heat shock response may provide a disease-modifying therapy for AD and other neurodegenerative diseases. Since pharmacologic inhibition of Hsp90 can induce the heat shock response, it has been proposed that Hsp90 inhibitors may offer a treatment option for AD. Thus, restoring Hsp70 levels upon Hsp90 inhibition can provide a beneficial effect against multiple aspects of AD pathogenesis (Luo et al., 2010). For example, in amyloid pathogenesis, overexpression of Hsp70 and Hsp90 has been shown to decrease Aβ aggregation (Evans et al., 2006), reduce Aβ-mediated neuronal toxicity, and appears to enhance the chaperone-mediated clearance of amyloid precursor protein (APP) and its amyloidgenic Aβ derivatives (Kumar et al., 2007). In tauopathy, increased levels of Hsp70 can inhibit tau aggregation, which promotes tau solubility and microtubule-binding

ability (Dou et al., 2003; Luo et al., 2007; Patterson et al., 2011). In addition, the overexpression of Hsp70 exhibits anti-apoptotic properties by increasing Bcl-2 levels and lowering the inflammatory response by reducing the production of matrix metalloproteinases (Brown, 2007). Since Hsp90 is also capable of maintaining mutated proteins, such as tau, in a folded and partially activated state, degradation may be prevented and may prolong insults to the cell. Therefore, elevated Hsp90 levels may exert negative effects. Fortunately, independent of HSF-1 activation, Hsp90 inhibition can reduce protein levels of kinases that contribute to tau hyperphosphorylation (Luo et al., 2007, 2010; Salminen et al., 2011). GSK3β, CDK5, and Akt are well-known kinases that are responsible for the phosphorylation of tau and are also Hsp90-dependent substrates. p35 and its cleavage product p25 are neuronal proteins that activate CDK5 and are also dependent upon Hsp90 for their activity. In addition, mutant tau is stabilized by interactions with Hsp90. Consequently, through Hsp90 inhibition, degradation of these kinases appears to reduce the amount of hyperphosphorylated tau, as well as to direct degradation of pathogenic tau species. In addition, Hsp90 exists as a heteroprotein complex that exhibits high affinity for inhibitors in affected areas of AD brain (Dickey et al., 2007), similar to the Hsp90 multiprotein complexes found in cancer cells. As oncoproteins develop dependence upon Hsp90, aberrant Tau and hyperphosphorylated tau, not-wild-type tau, also require Hsp90 for their stability (Luo et al., 2007). Therefore, Hsp90 inhibition may result in selective degradation of aberrant proteins that contribute to the pathogenicity of AD, while providing a cytoprotective response through induction of the heat shock response. It is worth pointing out that unlike normal Hsp90 client proteins, which are largely partially folded kinases and transcription factors, tau, as an intrinsically disordered protein, is expected to behave in a different manner.

III. Potential Therapeutic Effects of Hsp90 Inhibitors in Alzheimer's Disease

A. Hsp90 N-Terminal Inhibitors

Hsp90 inhibitors have been developed for cancer treatment based on the fact that inhibition of the Hsp90 protein folding machinery results in simultaneous disruption of multiple oncogenic pathways that are critical to malignant growth and proliferation (Biamonte et al., 2010; Blagg & Kerr, 2006; Kim et al., 2009). Geldanamycin (GDA, Fig. 3) was the first natural product inhibitor of Hsp90 identified, and this opened the door to an entirely new area of anticancer research. GDA continues to serve as a small molecule probe for investigation of Hsp90-dependent pathways for a number

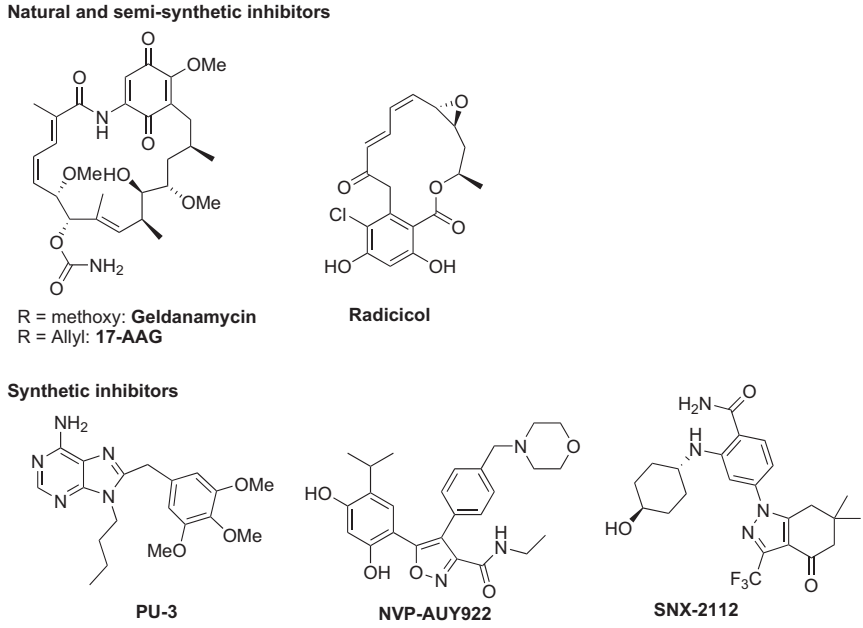

FIGURE 3 Structures of Hsp90 N-terminal inhibitors.

of disease states. Radicicol (RDC) was subsequently discovered as an Hsp90 inhibitor shortly after GDA, and both GDA and RDC have been shown to competitively bind to the N-terminal ATP-binding site. Due to the metabolically labile epoxide moiety, RDC was inactive *in vivo* and this prevented a thorough clinical evaluation. Synthetic Hsp90 inhibitors that possess purine (PU-3), isoxazole (NVP-AU922), and indazol-4-one (SNX-2112) scaffolds were designed to bind to the same ATP-binding pocket as both GDA and RDC. Analogues derived from these scaffolds are currently under investigation in clinical trials for the treatment of cancer. Unfortunately, the prosurvival heat shock response is always a complicating factor, and is observed at the same concentration needed to induce client protein degradation. This property is manifested by all Hsp90 N-terminal inhibitors and may ultimately compromise their therapeutic potential. Although the prosurvival heat shock response is not desired for the treatment of cancer, it may exhibit promising activities in the management and treatment of neurodegenerative diseases such as AD.

1. GDA and 17-AAG

Since the late 1990s, GDA has been known to induce the prosurvival heat shock response through Hsp90 inhibition, which disrupts HSF-1 binding (Zou et al., 1998). In 2001, Wanker demonstrated that upon GDA administration, dissociation of the HSF-1–Hsp90 complex readily ensued

and resulted in upregulation of Hsp40, Hsp70, and Hsp90. Not surprisingly, these conditions led to reduced huntingtin aggregation in a cell culture model of Huntington's disease (Sittler et al., 2001). The authors suggested that GDA-induced dissociation of the HSF-1–Hsp90 complexes, followed by a cooperative mechanism between Hsp40 and Hsp70 to reduce aggregation, and that Hsp90 itself appears not to be involved (Sittler et al., 2001). In support of this observation, Bonini and coworkers reported that direct expression of Hsp70 in an *in vivo* model suppressed α-synuclein neurotoxicity in a *Drosophila* model of Parkinson's disease (Auluck et al., 2002). Following these discoveries, Dou and coworkers demonstrated a similarly protective role for the heat shock response in a cellular model of AD. Upon administration of GDA at low concentrations, elevated levels of Hsp70/90 were observed that correlated directly with decreased tau aggregates and increased levels of soluble and microtubule-associated tau. Total tau levels were not altered in these studies (Dou et al., 2003). Contrary to Wanker's observation, Dou and coworkers demonstrated that suppression of either Hsp70 or Hsp90 by siRNA significantly reduced microtubule-associated tau and increased aggregated tau simultaneously, indicating that both chaperones are necessary for tau solubility and tau binding to microtubules (Dou et al., 2003).

17-AAG is a semisynthetic derivative of GDA that exhibits improved AMDE (absorption, distribution, metabolism, and excretion) properties and less toxicity. Knockdown of HSF-1 abolishes induction of the heat shock response in the presence of 17-AAG, indicating that the therapeutic effect manifested by 17-AAG is mediated by HSF-1, similar to GDA (Waza et al., 2005). Along with mild heat shock induction, 17-AAG induces selective degradation of the mutant androgen receptor (AR), an Hsp90-dependent client protein and pathogenic gene product in spinal and bulbar atrophy (SBMA), without significantly effecting the wild-type AR (Waza et al., 2005, 2006). GDA and its derivatives most likely exert their activity via two mechanisms: one which occurs through induction of the cytoprotective heat shock response, and the other which directs pathogenic proteins to degradation. However, GDA, 17-AAG, and their related derivatives may have limited applications in the treatment of neurodegenerative diseases such as AD, because the concentration needed to induce the HSR is similar to that needed to induce client protein degradation. The latter is likely to cause detrimental side effects and substantially narrow the therapeutic window. Moreover, GDA and its derivatives also exhibit limited solubility and poor blood–brain barrier penetration.

2. *Purine Derivatives*

PU scaffold derivatives, including PU-3, represent the first class of synthetic Hsp90 inhibitors (Chiosis et al., 2001). Although PU analogues were initially designed for cancer treatment, they bind to the Hsp90 N-terminus in

a manner similar to GDA, suggesting these compounds may also exhibit potential for the treatment of AD and other neurodegenerative diseases. The application of purine derivatives for AD has focused primarily on tauopathies, including aberrant tau phosphorylation. Rationale for Hsp90 modulation as a method to treat tauopathies is based upon Hsp90 function, which normally allows for the accumulation of abnormal tau species. However, inhibition of Hsp90 could function to reduce/clear aberrant tau and thus rescue neuronal cells from this toxic insult. Luo and coworkers showed that Hsp90 complexes regulated the stability of p35, a neuronal protein that is responsible for activation of CDK5—the kinase that phosphorylates tau, as well as mutant tau species (Luo et al., 2007). In cellular models of tauopathy, inhibition of Hsp90 upon the administration of PU24FCl (Fig. 4) caused degradation of p35 and resulted in decreased levels of mutant tau. Wild-type tau remained unscathed, along with several kinases and phosphatases that regulate normal tau activity, suggesting that selective degradation of pathogenic proteins is achievable through Hsp90 inhibition (Luo et al., 2007). In fact, administration of a single dose of PU-DZ8 (75mg/kg) to a tau transgenic mouse resulted in significant reductions of mutant tau and p35 levels, and a marked reduction in aggregated and hyperphosphorylated tau (Luo et al., 2007). The expression of prosurvival proteins, Akt and Raf-1, were not affected by PU-DZ8 in this mouse model, further demonstrating the potential selectivity afforded by these purine inhibitors. Interestingly, PU-DZ8 can accumulate to a concentration of ~700nM within 4h and can maintain this pharmacologically relevant dose for 12h, suggesting this molecule can pass the blood–brain barrier. (Taldone & Chiosis, 2009). Similarly, EC102, a structurally related purine analogue, was shown to cross the blood–brain barrier and accumulate in the brain at a relevant concentration within 1h at a dose of 200mg/kg per day for 7 days, without producing detectable toxicity. EC102 demonstrated the ability to promote degradation of only aberrant phosphorylated tau, without affecting normal tau in transgenic mouse models of AD, similar to PU-DZ8 (Dickey et al., 2007). Interestingly, EC102 exhibited a 1000-fold greater affinity for Hsp90 from affected areas of the brain, including the temporal cortex, suggesting that Hsp90 inhibitors may selectively act on stressed cells, while sparing normal

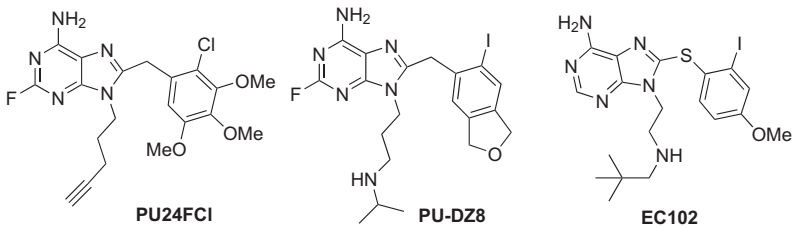

FIGURE 4 Structures of Hsp90 inhibitors with purine scaffolds.

neurons, a rationale similar to that for use of Hsp90 inhibitors as cancer chemotherapeutics. Not surprisingly, these PU analogues also lead to induction of the heat shock response under these concentrations, enabling the activation of prosurvival pathways. Collectively, these purine analogues demonstrate therapeutic efficacy, a good therapeutic index, and the ability to cross the blood–brain barrier, which supports their continued development for the treatment of AD.

B. Hsp90 C-Terminal Inhibitors

The C-terminal domain of Hsp90 is responsible for the homodimerization of Hsp90, which plays a key role in maintaining its biological function. The MEEVD domain located at the C-terminus is responsible for association with cochaperones and immunophilins that contain a TPR-recognition sequence, such as Hsp70–Hsp90 organizing protein (HOP) and FK506 binding protein 52 (FKBP52), which facilitate the loading of client proteins onto Hsp90 (Blagg & Kerr, 2006). This region also houses a nucleotide binding region that appears to allosterically regulate N-terminal ATPase activity. Therefore, small molecules that inhibit the Hsp90 C-terminus may disrupt cochaperone (such as HOP) binding to Hsp90, and subsequently block the loading of client proteins, resulting in their degradation through the UPS.

1. Novobiocin and Its Analogues

Novobiocin (NB, Fig. 5), a natural product isolated from *streptomyces* strains, exhibits antimicrobial activity by binding to the DNA gyrase ATP-binding pocket (Hooper et al., 1982). In 2000, NB was identified as the first Hsp90 C-terminal inhibitor, and provided a new opportunity for Hsp90

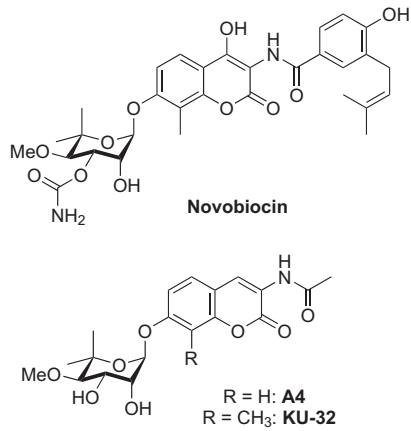

FIGURE 5 Structures of novobiocin and its derivatives.

modulation (Marcu et al., 2000). Upon the administration of NB, various Hsp90 client proteins including Raf-1, mutated p53, v-Src, and Her2 underwent degradation in a manner similar to that observed with GDA and radicicol. However, induction of the heat shock response was not observed (Marcu et al., 2000). In subsequent studies, Ovsenek examined the effect of NB on HSF-1 activity in Xenopus oocytes, alongside GDA (Conde et al., 2009). They demonstrated that oocytes treated with NB followed by heat shock decreased HSF-1 DNA binding and transcriptional activity in a dose-dependent manner. The co-immunoprecipitation analyses showed that in the presence of novobiocin, Hsp90 associated with both monomeric and trimerized HSF-1. In contrast, upon GDA administration, a dose-dependent increase in unbound HSF-1 was observed following submaximal heat shock. Upon the combination of NB (1mmol) and GDA (1μM), a decrease in HSF-1 activation was observed, similar to that which is common upon treatment of NB alone. These results suggest that the Hsp90 C-terminal binding site exhibits significant control over the N-terminal ATP-binding site.

Since NB exhibits low efficacy (~700μM in SKBr3 cells) as an Hsp90 inhibitor, extensive structural modifications to NB have been pursued with the goal of identifying molecules that manifest increased potency. A-4, a small molecule analogue of NB, was shown to exhibit excellent neuroprotective properties without observable toxicities at 100μM (Ansar et al., 2007). Pretreatment of embryonic primary neurons with A-4 significantly alleviated Aβ-induced toxicity in a dose-dependent manner with an EC50 of ~6nM. Hsp90 and Hsp70 expression paralleled neuroprotective activity in this Aβ-induced toxicity model for AD. Furthermore, A4 is not a substrate for the P-glycoprotein pump, and demonstrates a time-dependent linear transport across a brain microvessel endothelial layer, indicating its potential to cross the blood–brain barrier. KU-32, a derivative of A-4, exhibits ~10-fold increased neuroprotective activity, and is currently under preclinical development (Lu et al., 2009; Matts et al., 2011).

2. *AEG3482*

The loss of neuronal function is one characteristic of AD, suggesting that inhibition of neuronal cell death pathways may delay or halt progression of these diseases (Gallo, 2006). Activation of the c-Jun N-terminal kinase (JNK) signaling pathway is a central event during neuronal apoptosis, which is observed in both mouse models and pathological specimens from AD brain. Hsp70 has been shown to bind JNK and suppress its activity, which can reduce the number of neuronal cells that undergo apoptosis (Sherman et al., 2000). AEG3482 (Fig. 6), an Hsp90 inhibitor, demonstrated that Hsp70 induction can block apoptosis caused by the p75 neurotrophin receptor (p75NTR) or its interacting partner, NRAGE, through the blockade of proapoptotic JNK activation

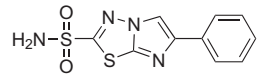

FIGURE 6 Structure of AEG3482.

(Salehi et al., 2006). Geldanamycin has not been shown to compete with AEG3482 for Hsp90 binding. AEG3482 exhibits no effect on Hsp90N-terminal ATPase activity, suggesting AEG3482 does not bind to this pocket. The prosurvival kinase, Akt, was not affected upon AEG3482 treatment. AEG3482 demonstrated that Hsp70 induced by Hsp90 inhibitors can block the JNK activation and rescue the neurons from apoptosis.

3. *ITZ-1*

ITZ-1 (Fig. 7) is a imidazo[5,1-c][1,4]thiazine derivative that was identified as a chondroprotective agent during an anti-osteoarthritis drug screening program (Kimura et al., 2009). It inhibits interleukin (IL)-1β-induced cartilage degradation both *in vitro* and *in vivo*, and suppresses nitric oxide–induced death of human articular chondrocyte by selectively inhibiting IL-1β induced ERK activation without affecting p38 kinase and JNK activation. The mechanism of action appears to result from binding to the C-terminus of Hsp90, and without disruption of Hsp90's ATPase activity (Kimura et al., 2010). ITZ-1 mediated Hsp90 inhibition induces HSF-1 activation, and the subsequent heat shock response comparable to GDA. Additionally, ITZ-1 causes mild Raf-1 degradation when compared to GDA, and is ~1000-fold less potent toward the degradation of Hsp90 client proteins, such as glucocorticoid receptor (GR), Akt, epidermal growth factor receptor (EGFR), and receptor interacting proteins (RIP) (Kimura et al., 2010). Although there is no AD-related research on ITZ-1, its strong HSF-1 induction, lack of client-protein degradation, and low cytotoxicity suggest it may be a viable candidate for AD and other neurodegenerative diseases.

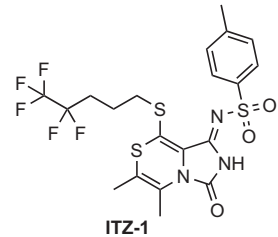

FIGURE 7 Structure of ITZ-1.

C. Agents That Disrupt Protein–Protein Interactions

1. Celastrol

Celastrol (Fig. 8) is a natural product derived from the *Celastraceae* family of plants (Kutney et al., 1981). Extracts containing celastrol have been used for the treatment of fever, joint pain, and edema without evidence of carcinogenicity or other limiting side effects (Allison et al., 2001). Recently, celastrol was identified from a panel of 1040 existing drugs as a neuroprotective agent through a collaborative drug screen that targeted the identification of small molecules for the treatment of neurodegenerative diseases. In contrast to Hsp90 N- and C-terminal inhibitors, Sun and coworkers demonstrated that celastrol disrupts the interaction between Hsp90 and the cochaperone, Cdc 37. The celastrol binding site appears to be located in the N-terminal region of Hsp90 and does not prevent ATP from binding. (Zhang et al., 2008; T. Zhang et al., 2009). Dickey and coworkers recently showed that Cdc37 worked in conjunction with Hsp90 to regulate various aspects of tau pathogenesis. They demonstrated that Cdc37 knockdown reduced the levels of CDK5 and Akt significantly, but had little effect on GSK3β and Mark2 (Jinwal et al., 2011). Since Hsp90 requires the cochaperone Cdc37 to load clients onto the Hsp90 superchaperone complex, disruption of Hsp90/Cdc37 interactions by celastrol appears to induce selective degradation of Hsp90 clients, such as CDK5 and Akt, which appear to be Cdc37 dependent. Recent studies suggest that the beneficial effect of celastrol on AD may also be associated with HSP induction. In 2004, Morimoto and coworkers demonstrated that administration of celastrol at 3μM concentrations resulted in HSF-1/DNA binding interactions similar to those induced by heat shock in HeLa and SH-SY5Y neuronal cell lines (Westerheide et al., 2004). It was previously reported that neurons found in various differentiated states can be resistant to HSP induction following conventional heat shock, both *in vivo* and *in vitro*. However, in both differentiated human neurons and rodent neurons, celastrol-induced neuroprotective HSPs (Brown, 2007). However, it remains unknown whether heat shock induction is a robust process in old cells. In animal models of AD, celastrol led to improved memory, learning, and

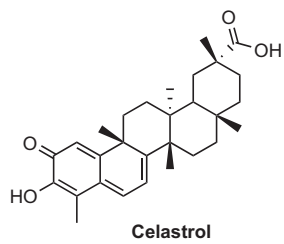

FIGURE 8 Structure of celastrol.

psychomotor activity at 7μg/kg (Allison et al., 2001). Taken together, the therapeutic benefits of Hsp90 modulation with celastrol for the treatment of AD may be related to the induction of chaperone and disruption of the Hsp90–Cdc37 interaction.

2. *Gedunin*

Gedunin (Fig. 9) is a tetranortriterpenoid that can be extracted from the Indian neem tree (*Azadirachta indica*), which has been historically used in homoeopathic medicine (Subapriya & Nagini, 2005). It demonstrates antiparasitic (Misra et al., 2011; Omar et al., 2003), antisecretory (Lakshmi et al., 2010), antifungal (Sundarasivarao et al., 1977), anticancer (Brandt et al., 2008; Kamath et al., 2009), and neuroprotective activities (B. Zhang et al., 2009). Both its structure and its traditional applications are similar to those of celastrol, and indicate a common mechanism of action (Subapriya & Nagini, 2005). Not surprisingly, gedunin and celastrol are both Hsp90 inhibitors that were identified through connectivity map screening (Hieronymus et al., 2006)—a technology that utilizes a systematic tool for evaluating the connections between disease, genetic perturbations, and drug action (Brandt & Blagg, 2009; Lamb, 2007; Lamb et al., 2006). Upon completion of this screening, gedunin and celastrol were shown to produce highly similar gene expression profiles to those produced by GDA, 17-AAG, 17-DMAG, and radicicol, all of which were previously identified N-terminal inhibitors. However, celastrol and gedunin were shown to modulate Hsp90 machinery in a mechanism distinct from N-terminal inhibitors (Hieronymus et al., 2006). Both compounds were shown to disrupt interactions between Hsp90 and the cochaperone, Cdc37. Interestingly, during a high content screening (HCS) study to identify HSF-1 amplifiers, three gedunin derivatives, but not gedunin itself, were associated with a strong increase in HSP70 levels. Furthermore, these compounds exhibited potent cytoprotective activity in an MG-132 (a 26S proteasome inhibitor)-induced protein misfolding neuronal cell model, as well as a cellular model for Huntington's disease (B. Zhang et al., 2009).

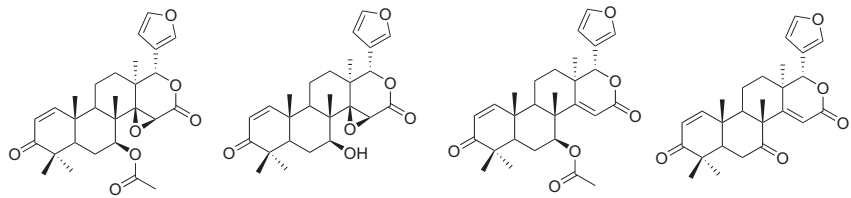

FIGURE 9 Structure of gedunin and its derivatives.

D. Polyphenols

Polyphenols refer to a diverse class of natural product–containing phenols that can be isolated from plants (Kim et al., 2010; Queen & Tollefsbol, 2010; Singh et al., 2008). Due to their excellent antioxidant and anti-inflammatory properties, polyphenols have a long history of applications against oxidative stress, chronic inflammation, and the buildup of toxins—all three of which represent major factors associated with aging (Queen & Tollefsbol, 2010). Their neuroprotective activity is among the most investigated areas of polyphenol research, and studies have suggested that long-term daily intake of these antioxidants may prevent or delay the onset of AD and other neurodegenerative diseases (Kim et al., 2010). Epigallocatechin-3-gallate (EGCG), curcumin, and silybin are representative polyphenols that exhibit beneficial activities in AD. Extensive investigation of these polyphenols has resulted in the discovery of numerous mechanisms for their biological activities, including Hsp90 modulation.

1. EGCG

The neuroprotective role of catechins is becoming increasingly recognized. EGCG (Fig. 10) is one of the most abundant catechins and can be readily extracted from green tea. The mechanism of action for EGCG in AD is a central focus of catechin research. EGCG alters APP processing by enhancing nonamyloidgenic α-secretase cleavage, but not the competing β- and γ-secretase cleavages that lead to amyloidogenic Aβ peptides (Fernandez et al., 2010). Increasing evidence suggests that Aβ oligmers might be the most toxic forms of this peptide, and EGCG can act like a small molecule chaperone by directly binding to unfolded polypeptides and shifting their aggregation away from amyloid oligomers and fibrils and redirecting them toward unstructured and nontoxic spherical oligomers (Ehrnhoefer et al., 2008). Recently, Gasiewicz and coworkers identified EGCG as an Hsp90 inhibitor that acts by binding to Hsp90 at or near the C-terminus as demonstrated by proteolytic footprinting, immunoprecipitation, and an ATP-agarose pull-down assay (Palermo et al., 2005; Yin et al., 2009). EGCG

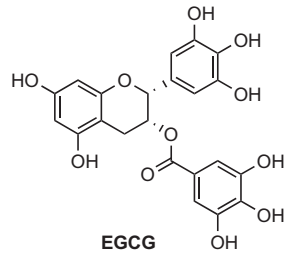

FIGURE 10 Structure of EGCG.

also disrupts the association between Hsp90 and cochaperones, such as p23 and Hsc70, and it also induces Hsp90 client protein degradation (Li et al., 2009). Interestingly, EGCG has been reported to reduce the expression of Hsp70 and Hsp90 without affecting other Hsps, through suppression of HSF-1 and HSF-2 in the MCF-7 cell line (Tran et al., 2010), and thus, resembling the effects manifested by NB.

2. *Curcumin*

Curcumin (Fig. 11) is a biologically active natural product isolated from Indian plant turmeric, *Curcuma longa*— a dried rhizome powder that is widely used in curries (Singh et al., 2008). Traditionally, it has been used for the treatment of wounds, inflammation, and tumors, suggesting that it exhibits anti-inflammatory, antioxidant, and antitumor activities (Marathe et al., 2011; Singh et al., 2008). The high consumption of curcumin is thought to be responsible for the lower incidence of AD among senior Indians when compared to those residing in the United States (Ganguli et al., 2000). The potential application of curcumin to AD is well recognized (Hamaguchi et al., 2010; Zhou et al., 2011) and is currently being investigated in several clinical trials (Baum et al., 2008; Hatcher et al., 2008). At the molecular level, curcumin exhibits anti-amyloidogenic activity by preventing the aggregation of fresh amyloid-β and dissociating Aβ fibrils back into a momeric form in a dose-dependent manner. Similar to EGCG, it also behaves as a chemical chaperone and directly binds to Aβ monomers, and thus, preventing their polymerization (Ono et al., 2004). Curcumin has been shown to inhibit β-secretase and acetylcholinesterase, as well as Aβ-induced inflammation (Hamaguchi et al., 2010). In recent studies, curcumin has been shown to exhibit high affinity for Hsp90 during surface plasmon resonance (SPR) studies and induce the degradation of Hsp90 client proteins, including EGFR, Raf-1, Survivin, and CDK4 in squamous cell carcinoma A431 and mesothelioma STO cells (Giommarelli et al., 2010). Consistent with this observation, Blagg and colleagues determined that curcumin binds to purified recombinant Hsp90, inhibits Hsp90-dependent luciferase refolding, and disrupts Hsp90/Cdc37-dependent activation of the heme-regulated elF2α kinase in rabbit reticulocyte lysates. In addition, they showed that curcumin induces the degradation of Her2, Raf-1, and Akt at concentrations that parallel its antiproliferative activity. Although there is no

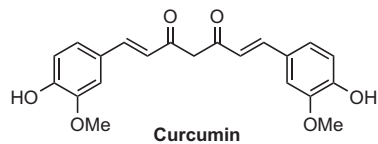

FIGURE 11 Structure of curcumin.

direct evidence showing Hsp90 modulation by curcumin in AD models, studies performed in cancer cells suggest that Hsp90 modulation by curcumin may be beneficial for AD.

3. *Silybin*

Silybin (Fig. 12) exists as a mixture of two diastereomers, A and B, in a nearly 1:1 ratio and is the active component of silymarin, a flavonolignan extract from the seed of milk thistle (*Silybum marianum*) (Abenavoli et al., 2010). It has been used traditionally for the treatment of liver and gallbladder disorders. More recently, silybin has been used clinically as an anti-hepatotoxic agent as well as a nutritional supplement to protect the liver from diseases associated with alcohol consumption and exposure to chemical and environmental toxins (Gazak et al., 2007). Because of its excellent antioxidant and anti-inflammatory activities, its potential use as an anti-amyloidogenesis was evaluated. Lu and coworkers found that silybin suppressed nitrotyrosine levels and inhibited the overexpression of iNOS and TNF-α mRNA in the hippocampus and amygdale induced by $A\beta_{25-35}$. In addition, silybin alleviated memory deficits resulting from $A\beta_{25-35}$ in several AD mouse models (Lu et al., 2009; Lu et al., 2009). Similar to curcumin, silybin was shown to exhibit Hsp90 inhibitory activity through Hsp90-dependent firefly luciferase refolding and Hsp90-dependent heme-regulated eIF2a kinase (HRI) activation assays. Consistent with Hsp90 inhibition, the administration of silybin also leads to Hsp90 client protein degradation (Zhao et al., 2011), implying the potential utility of silybin for treatment against AD.

IV. Conclusion

Because AD is an age-related disease, the incidence of disease is expected to increase at an unprecedented rate that parallels the aging population.

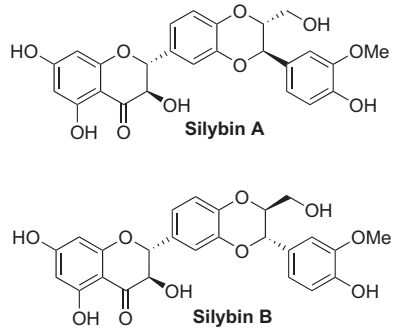

FIGURE 12 Structure of silybin.

Although extensive efforts have been made toward identification of a cure for this disease, the looming fate of AD victims is largely unchanged and the exact mechanisms for the onset of this disease remain largely unsolved. After 30 years of research, it has been determined that AD is a multimechanistic disease and that targeting one specific mechanism may not be sufficient. The heat shock response is a defensive mechanism that serves to maintain cell proteostasis and integrity. Unfortunately, this protective function appears to undergo derailment during the aging process. Pharmacological inhibition of Hsp90 is capable of restoring this function and can positively affect multiple AD hallmarks in both cellular and animal models. Consequently, Hsp90 modulation by small molecules may provide the much needed multifaceted approach toward managing this highly complex disease.

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Using Pittsburgh Compound B for *In Vivo* PET Imaging of Fibrillar Amyloid-Beta

Abstract

The development of Aβ-PET imaging agents has allowed for detection of fibrillar Aβ deposition *in vivo* and marks a major advancement in understanding the role of Aβ in Alzheimer's disease (AD). Imaging Aβ thus has many potential clinical benefits: early or perhaps preclinical detection of disease and accurately distinguishing AD from dementias of other non-Aβ causes in patients presenting with mild or atypical symptoms or confounding comorbidities (in which the distinction is difficult to make clinically). From a research perspective, imaging Aβ allows us to study relationships between amyloid pathology and changes in cognition, brain structure, and function across the continuum from normal aging to mild cognitive impairment (MCI) to AD; and to monitor the effectiveness of anti-Aβ drugs and relate them to neurodegeneration and clinical symptoms. Here, we will

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discuss the application of one of the most broadly studied and widely used Aβ imaging agents, **Pi**ttsburgh Compound-**B** (PiB).

I. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly and its prevalence is increasing at an alarming rate, with a worldwide prevalence estimated to quadruple over the next 50 years. AD is pathologically characterized by the presence of amyloid plaques, containing amyloid-β (Aβ), and neurofibrillary tangles (NFT), containing hyperphosphorylated tau, as well as significant loss of neurons and deficits in neurotransmitter systems. A growing consensus points to deposition of Aβ plaques as a central event in the pathogenesis of AD. This "amyloid cascade hypothesis" (Hardy & Allsop, 1991; Hardy & Higgins, 1992) states that overproduction of Aβ, or failure to clear this peptide, leads to AD primarily through amyloid deposition, which triggers the production of NFT, cell death and, ultimately, the clinical symptoms such as memory loss and cognitive impairment (Hardy et al., 1998). Further, the presence of Aβ in AD has been associated with synaptic loss (for review see Wilcox et al., 2011), which is significantly correlated with cognitive impairment in AD (DeKosky et al., 1996; Terry et al., 1991;). The single, most important piece of evidence for this "amyloid cascade hypothesis" of AD is the demonstration that at least five different mutations in the Aβ precursor protein (APP) gene on chromosome 21, all lying in or near the Aβ peptide region, cause early-onset AD (Hardy et al.,1998; Price & Sisodia, et al., 1998; Tanzi et al., 1996). Further genetic support for the amyloid cascade hypothesis comes from the finding that the most common form of early-onset, autosomal dominant, familial AD (eoFAD) (the chromosome 14 mutations) is caused by mutations in the presenilin-1 (*PS1*) gene which codes for a protein that is a component of the "γ-secretase" enzyme complex responsible for C-terminal cleavage of Aβ from APP (Xia et al., 2000).

II. Rationale for Studying Amyloid Deposition

Definitive diagnosis of AD relies on the demonstration of sufficient amounts of Aβ plaques and NFT in autopsy brains (Mirra et al., 1991). Imaging Aβ thus has many potential clinical benefits: early or perhaps preclinical detection of disease and accurately distinguishing AD from non-Aβ causes of dementia in patients with mild or atypical symptoms or confounding comorbidities (in which the distinction is difficult to make clinically). From a research perspective, imaging Aβ allows us to study relationships between amyloid, cognition, and brain structure, and function across the continuum from normal aging to AD; and to monitor the biological effects

of anti-Aβ drugs and relate them to effects on neurodegeneration and cognition. Here, we will discuss the application of one of the most broadly studied and widely used agents, **P**ittsburgh Compound-**B** (PiB).

III. General Properties of the Aβ Imaging Tracer, PiB

PiB (also known as [^{11}C]6-OH-BTA-1 or [N-methyl-^{11}C]2-(4'-methylaminophenyl)-6-hydroxybenzothiazole (Mathis et al., 2003)) is a thioflavin-T (ThT) derivative, a small molecule known to bind amyloid proteins aggregated into a beta-pleated sheet structure (Levine 1995). Figure 1 demonstrates the steps in development of PiB from ThT. The first step removed the methyl group from the positively charged quaternary heterocyclic nitrogen of the benzothiazolium group of ThT, yielding a compound called 6-Me-BTA-2. This alteration produced increased brain entry of the compound and improved the Aβ binding affinity and highly decreased the NFT binding affinity relative

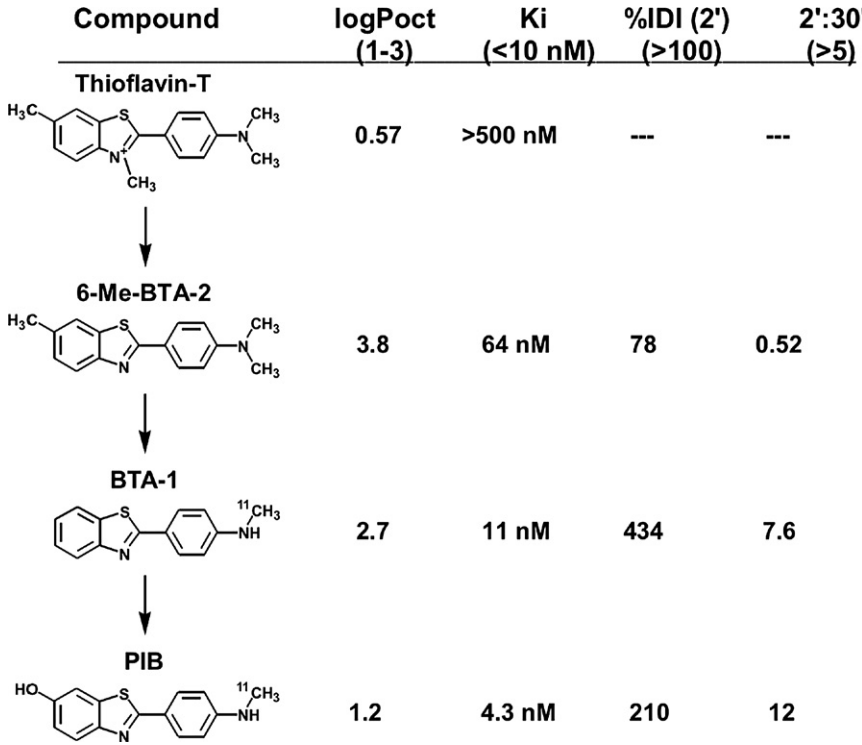

FIGURE 1 Chemical structures, lipophilicity ($logP_{oct}$), Aβ binding affinity (Ki), and brain entry [%Injected Dose Index (%IDI) or (%ID × g body weight)/g brain weight] and brain clearance (2':30' ratio) of thioflavin-T, PiB and intervening derivatives. Numbers in parentheses indicate targets for each parameter.

to the parent compound ThT. The inhibition constant (K_i, a measure of binding affinity closely related to the K_d (Bennett & Yamamura, 1985)) of 6-Me-BTA-2 for fibrillar Aβ was nearly ten times lower than ThT, although it did not reach the desired binding affinity of <10 nM (Fig. 1). Additionally, the brain clearance of 6-Me-BTA-2 from normal brain was very poor and brain levels actually increased 2-fold over 30 min; therefore, two additional methyl groups were removed from 6-Me-BTA-2, creating a compound known as BTA-1, which showed significantly better Aβ affinity, brain entry, and clearance (Mathis et al., 2003). However, the 6-hydroxy derivative of BTA-1 (6-OH-BTA-1 or PiB) had a better Aβ affinity, with a K_i of 4.3 nM (surpassing the initial goal of 10 nM) and a better normal brain clearance, with a 2':30' ratio of 12 (normal brain clearance $t_{1/2}$ ~7.9 min) and was used for further human studies (Fig. 1).

IV. Early Human PiB Studies

The first human positron-emission tomography (PET) imaging studies with PiB were a collaboration between the University of Pittsburgh and Uppsala and Karolinska Universities (Engler et al., 2002; Klunk et al., 2004). This study included 16 AD patients, six elderly age-matched controls, and three young controls, chosen because of the likelihood that most would be amyloid negative. The healthy control (HC) subjects showed rapid entry and

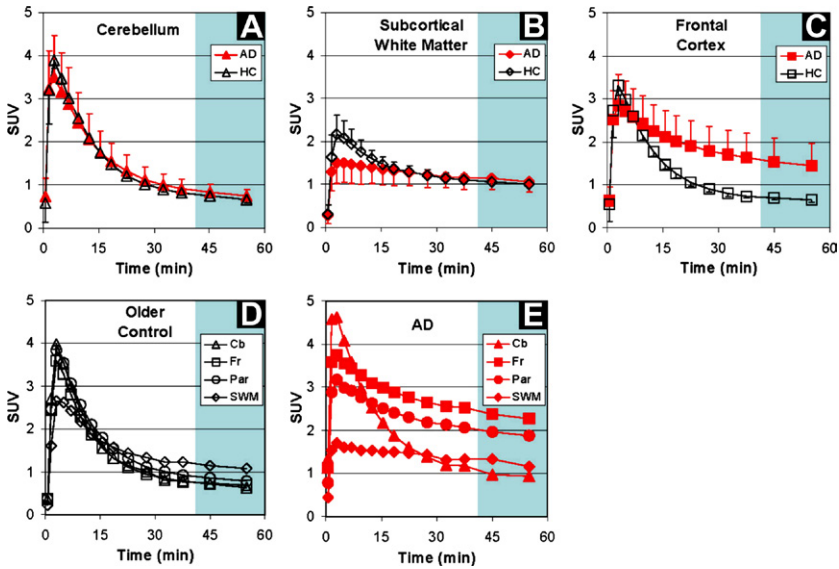

FIGURE 2 Standardized uptake values (SUV; 1.0 SUV = 0.10 %IDI) demonstrating brain entry and clearance of PiB in varying brain regions. For color version of this figure, the reader is referred to the online version of this book.
(from Klunk et al., 2004)

clearance of PiB from all cortical and subcortical grey matter areas, including the cerebellum (Fig. 2). Nearly identical uptake and clearance of PiB was seen in the cerebellum of HC and AD groups (Fig. 2A), an area of the brain known to have few fibrillar Aβ deposits. Subcortical white matter showed relatively lower entry and slower clearance in both HC subjects and AD patients compared to grey matter areas (Fig. 2B). However, in AD patients, markedly increased PiB retention was observed in brain areas known to contain high levels of amyloid plaques when compared to HC subjects, including brain regions such as parietal and frontal cortices (Figs. 2C–E) (Arnold H et al., 1991; Thal, Rub, Orantes, & Braak, 2002).

The pattern of PiB retention was quite different in AD patients compared to the HC subjects (Fig. 3). PiB retention in AD patients was generally most prominent in cortical areas and lower in white matter areas, in a manner most consistent with postmortem studies of Aβ plaques in the AD brain (Thal et al., 2002). PiB retention was broadly observed in frontal cortex in AD, but also was observed in precuneus/posterior cingulate, temporal, and parietal cortices. The occipital cortex and lateral temporal cortex were also significantly affected with a relative sparing of the mesial temporal areas. Significant striatal PiB retention was also observed, consistent with previous reports of extensive Aβ deposition in the striatum of AD patients (Braak & Braak, 1990; Brilliant et al., 1997; Suenaga et al., 1990; Wolf et al., 1999). PiB images from HC subjects showed little or no PiB retention in cortical areas, and the accumulation of PiB in white matter was the same in AD patients and HC subjects (Fig. 2B).

In the initial PiB-PET study three AD subjects displayed cortical PiB retention at the level of HC subjects – this is not a particularly surprising finding when one considers previous reports from postmortem studies that some people clinically diagnosed with AD do not have Aβ deposits at autopsy (Haroutunian et al., 1998; Price and Morris, 1999). Indeed, these three AD patients performed well on the mini-mental status exam and showed no significant cognitive deterioration over the 2–4 year follow-up period after the

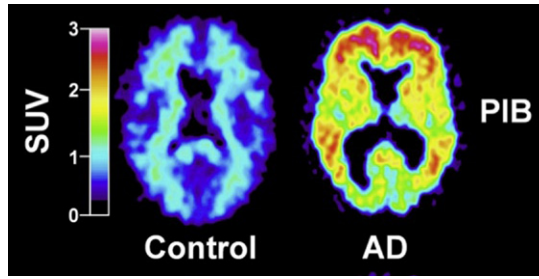

FIGURE 3 PIB standardized uptake value images demonstrate a marked difference between PIB retention in AD patients and HC subjects. For color version of this figure, the reader is referred to the online version of this book.
(from Klunk et al., 2004)

PiB study (i.e., MMSE remained 28–29) while the AD patients with significant PiB retention showed deterioration typical of clinical AD. Additionally, in the elderly HC group, the oldest subject (76 y/o) consistently showed the highest cortical PiB retention, consistent with postmortem studies identifying elderly HC subjects with significant amyloid deposits (Bennet et al., 2006). It was recognized very early that it would be critical to longitudinally follow PiB retention in these discordant subjects (i.e., clinical AD-absent PiB or HC-significant PiB) in order to gain insight into the natural history of Aβ deposition and the role it may (or may not) play in cognitive decline and clinical AD.

The initial PiB study was followed by a 2-year follow-up study which examined the clinical history of three PiB-negative [PiB(–)] AD patients and the PiB-positive [PiB(+)] HC subject (Engler et al., 2006). At 2-year follow-up, all three of the PiB-negative AD subjects were reclassified as mild cognitive impairment (MCI)—although it is not clear if this was by clinicians blinded to the PiB-PET results. The single PiB(+) HC subject showed no change in cognition or regional cerebral metabolic rate of glucose (rCMRglc), measured with Flurodeoxyglucose (FDG)-PET over the follow-up period, and little increase in PiB retention. These data suggest that the PET result was either false positive, if PiB retention followed a fairly rapid course, or true positive if PiB retention began long before clinical symptoms and followed a fairly lengthy course. These original studies provided a landmark description of the natural history of Aβ deposition in living subjects, and were later confirmed by additional studies using PiB in AD patients and cognitively normal subjects (Archer et al., 2006; Buckner et al., 2005; Edison et al., 2006; Fagan et al., 2006; Fagan et al., 2007; Jack et al., 2009; Kemppainen et al., 2006; Lopresti et al., 2005; Mintun et al., 2006; Nelissen et al., 2007; Price et al., 2005; Pike et al., 2007; Rabinovici et al., 2007; Rowe et al., 2007; Ziolko et al., 2006).

V. Amyloid Imaging and Apolipoprotein-E Genotype

Apolipoprotein E (ApoE) is a 299 amino-acid protein involved in lipid transport and metabolism in the periphery and in brain. ApoE plays a key role in neuronal maintenance and repair (for review see Mahley et al., PNAS 2006). The *ApoE* gene, found on chromosome 19, has three common isoforms: ε3 (allele frequency 65–70%), ε2 (5–10%), and ε4 (15–20%). The ε4 allele (ApoE4) is by far the strongest genetic risk factor for sporadic AD, associated with a 3-fold increased risk in heterozygotes and up to a 15-fold increased risk of AD in homozygotes (Farrer et al., 1997), while ApoE2 may be protective. ApoE4 has been implicated in multiple aspects of AD pathogenesis, including Aβ fibrillization and clearance (Mahley et al., 2006). Autopsy studies have demonstrated an increased likelihood of AD pathology in cognitively normal individuals who are ApoE4 carriers (Kok et al., 2009).

Similarly, PiB-PET studies have found that ApoE4 genotype is associated with higher PiB retention in cognitively normal elderly in a dose-dependent manner (Reiman et al., 2009, Morris et al., 2010), and ApoE4 carriers are more than twice as likely to convert from PiB(–) to PiB(+) over time (Vlassenko et al., 2011). Conversely, ApoE2 has been associated with lower PiB retention in normal elderly (Morris et al., 2010). MCI patients who are ApoE4 carriers consistently show higher PiB retention than MCI noncarriers, though this is at least in part because the presence of ApoE4 increases the likelihood that MCI symptoms are due to underlying AD (Kemppainen et al., 2007; Rowe et al., 2007). Findings in AD patients have been mixed, with some studies demonstrating increased PiB retention in ApoE4 carriers cross-sectionally (Drezga et al., 2008) and longitudinally (Grimmer et al., 2010), while other studies did not find differences between ApoE4 carriers and noncarriers in AD (Klunk et al., 2004; Rowe et al., 2007; Rabinovici et al., 2010). Similarly, ambiguous results have been reported in the AD postmortem literature (Berg et al., 1998; Gomez-Isla et al., 1996). Amyloid imaging will be helpful in further elucidating the links between ApoE, Aβ, neurodegeneration, and cognition across the AD continuum.

VI. Amyloid Imaging in Normal Controls

Several studies have now demonstrated PiB retention in cognitively normal controls. Depending on the site, reports have ranged from a proportion of 10–30% of normal elderly subjects with significant PiB retention [i.e., PiB(+)] (Aizenstein et al., 2008; Jack et al., 2008; Kantarci et al., 2012; Klunk et al., 2004; Mintun et al., 2006; Mormino et al., 2009; Mormino et al., 2011; Pike et al., 2007; Reiman et al., 2009; Rowe et al., 2010; Villemagne et al., 2008). This wide range likely depends on factors such as the age of the cohort, proportion of subjects carrying the ApoE4 allele, definition of "cognitively normal," and the threshold for defining amyloid-positivity. The relationship between increased PiB retention and cognition in the normal elderly has been difficult to define. It is apparent that among cognitively normal subjects, significant plaque load is not related to broad differences in cognitive performance between groups with and without significant PiB retention (Aizenstein et al., 2008; Jack et al., 2008; Mintun et al., 2006; Rowe et al., 2010). In other studies, an increase in PiB retention has been associated with poorer performance on episodic memory tests (Kantarci et al., 2012; Mormino et al., 2009; Pike et al., 2007; Villemagne et al., 2008). More consistently, PiB(+) cognitively normal individuals show, at a group level, "AD-like" changes in brain structure and network connectivity and activity (see PiB and MRI section, Section XIV: B, below). Most significantly, longitudinal studies have found that cognitively normal individuals with elevated PiB are at much higher risk for longitudinal cognitive decline and the emergence of clinically

significant cognitive impairment than PiB(–) age and education matched subjects (Morris et al., 2010; Resnick et al., 2010; Storandt et al., 2009; Villemagne et al., 2008; Villemagne et al., 2011a). These data have led to the hypothesis that, at least in many older individuals, PiB-positivity is a marker for preclinical AD (Sperling et al., 2011).

VII. Amyloid Imaging in MCI

In early studies of MCI subjects, PiB appeared to show a bimodal distribution, with 60–75% of subjects showing a typical, AD-like pattern and burden of PiB retention, while the remaining subjects showed levels typical of PiB(–) controls (Jack et al., 2008; Lopresti et al., 2005; Price et al., 2005; Rowe et al. 2007). Variations in PiB retention have also been explored when examining MCI subjects based on MCI subtype; subjects with nonamnestic MCI were much less likely to be PiB(+) than subjects with amnestic MCI, further suggesting that PiB may be superior to FDG in distinguishing MCI subtypes (Lowe et al., 2009; Pike et al., 2007). These studies have suggested that the nonamnestic MCI subtype may include depression or incipient dementia where Aβ deposition is not a feature [e.g., frontotemporal or vascular dementia (VaD)], or they may prove to be part of the 5–10% who have stable MCI, or the 20% who revert to apparent normality (Busse et al., 2006; Gauthier et al., 2006).

Longitudinal studies have suggested that MCI subjects with high PiB retention are much more likely to convert to AD than subjects with low PiB retention. In a study by Forsberg and colleagues (Forsberg et al., 2007), all 7 MCI-to-AD converters were amyloid-positive at baseline and 9 of the 14 nonconverters were amyloid-negative. In addition, none of the baseline PiB(–) MCI subjects converted to AD. This effect has also been observed in several subsequent studies, with MCI subjects with increased PiB retention showing much more frequent conversion to AD (Koivunen et al., 2011; Villemagne et al., 2011a; Wolk et al., 2009). Therefore, amyloid PET is likely to have a prognostic role in the clinical evaluation of MCI, by identifying subjects who have underlying AD pathophysiology and are therefore at high risk for further clinical decline (Albert et al., 2011).

VIII. Amyloid Deposition in Early-Onset, Autosomal Dominant, Familial AD

Roughly 1% of all AD cases are caused by single gene mutations that are transmitted in an autosomal dominant pattern with nearly 100% penetrance. Familial AD has been linked to mutations in presenilin-1 (*PS1*, chromosome 14, the most commonly involved gene), amyloid precursor protein

(APP, chromosome 21) or presenilin-2 (*PS2*, chromosome 1). All these mutations are thought to cause eoFAD by promoting the cleavage of APP to the proaggregatory $A\beta_{1\text{-}42}$ peptide (Hardy et al., 1998). In order to explore the natural history of preclinical amyloid deposition in people at high risk for AD, individuals with eoFAD have been evaluated in several studies. In the first PiB-PET study, subjects with two different *PS1* mutations were explored (Klunk et al., 2005). The *PS1* mutation carriers, both symptomatic and asymptomatic, showed a strikingly similar, focal amyloid deposition that appeared to begin in the striatum (Fig. 4). This is in contrast to early deposition of amyloid in nonmutation carriers, typically in the frontal cortex and the precuneus/posterior cingulate region but not in striatum.

These data have been extended to 49-year-old and 60-year-old siblings with autosomal dominant dementia and frequent cerebral amyloid angiopathy (CAA) and intracerebral hemorrhages due to an APP locus duplication (Remes et al., 2004; Rovelet-Lecrux et al., 2007). Similar to previous findings, PiB retention was highest in the striatum (up to 280% of the control mean) and the overall pattern of increased PiB retention was different from that seen in sporadic AD (Remes et al., 2007).

Theuns et al. (2006) reported widespread retention of PiB, typical of that observed in sporadic AD, in a 57-year-old patient (MMSE of 18) with

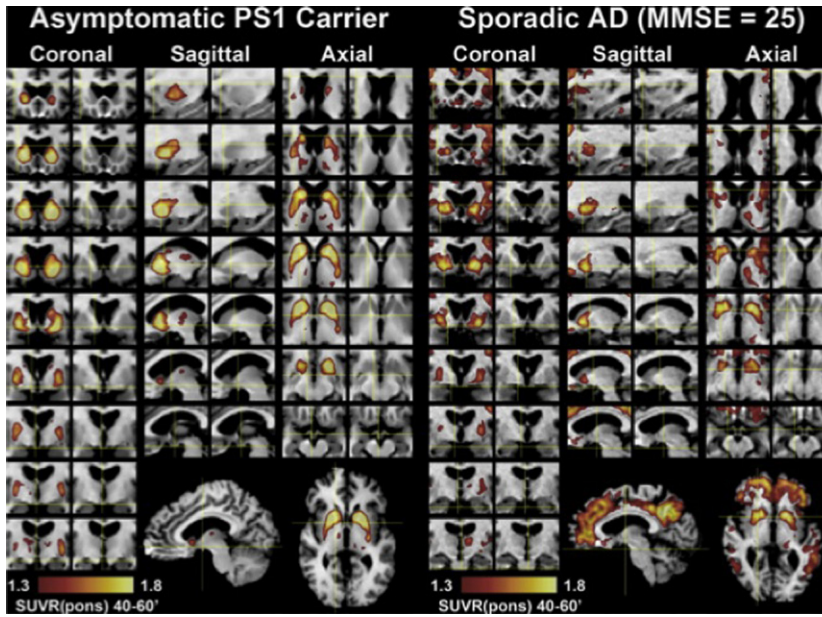

FIGURE 4 Detailed regional distribution of PiB retention in an asymptomatic PS1 carrier compared with a subject with sporadic AD. For color version of this figure, the reader is referred to the online version of this book.
(from Klunk et al., 2007)

a novel K724N mutation in the C-terminal intracytosolic fragment of APP. The subject showed no disproportionate PiB retention in the striatum. However, Villemagne et al. (2009), has demonstrated increased striatal PiB deposition in *PS1* and APP mutation carriers. Further, Pittsburgh investigators have shown a similar striatal PiB retention pattern in older nondemented subjects with Down syndrome (Handen et al., in press), while Landt et al. (2011) showed a typical AD PiB retention pattern in one older subject with Down syndrome. These early-onset forms of AD all share overproduction of Aβ (particularly the 42 amino acid form) as a proposed mechanism of Aβ deposition (Younkin, 1997), whereas decreased clearance might be more important in late-onset AD (Whitaker et al., 2003). It may be that the cellular milieu of the striatum is particularly prone to amyloid deposition under conditions of overproduction.

It has been reported that two genetic forms of AD, the Arctic APP mutation and the Osaka APP mutation, were found to have little PiB retention in the brains of mutation carriers—in contrast to subjects with late-onset AD. Interestingly, these mutations have been associated with enhanced formation of Aβ oligomers without Aβ fibril formation (Nilsberth et al., 2001; Tomiyama et al., 2008). The lack of PiB-PET signal in both the Arctic and Osaka mutations suggest that oligomeric Aβ, rather than fibrillar Aβ, plays a significant role in the cause of dementia symptoms observed in patients carrying these genetic mutations (Shimada et al., 2011; Scholl et al., in press; Tomiyama et al., 2008).

IX. Frontotemporal Dementia

Frontotemporal dementia (FTD) refers to a family of neurodegenerative disorders that preferentially affect the frontal and anterior temporal lobes (Rabinovici & Miller, 2010). Clinically, FTD presents with progressive changes in behavior and social-emotional function (in the behavioral-variant) or with decline in language in the semantic and nonfluent/agrammatic variants of primary progressive aphasia (Gorno-Tempini et al., 2011; Rascovsky et al., 2011). Histologically, FTD clinical syndromes are associated with a group of pathologies collectively referred to as frontotemporal lobar degeneration (FTLD). Inclusions in FTLD consist of tau, TDP-43, or (rarely) fused in sarcoma (FUS) proteins, but, significantly, do not include Aβ deposits (Mackenzie et al., 2010). AD and FTD can overlap clinically and anatomically, and misclassification rates of 10–40% are cited even at expert centers when clinical diagnosis during life is compared to postmortem findings (Alladi et al., 2007; Forman et al., 2006).

PiB-PET could be helpful in distinguishing AD and FTD, since amyloid plaques are a core feature of AD but are not part of the FTLD pathologic spectrum. Further, patients with FTD typically develop symptoms before

age 65 (Johnson et al., 2005), when the prevalence of AD and FTD is similar (Ratnavalli et al., 2002) and "age-related" amyloid deposits are less common (Morris et al., 2010). Several early case series demonstrated the utility of PiB-PET in distinguishing AD and FTD (Drzezga et al., 2008; Engler et al., 2007; Rabinovici et al., 2007; Rowe et al., 2007). In the largest series published to date, Rabinovici et al. tested the diagnostic performance of PiB-PET in distinguishing clinically diagnosed AD ($N = 62$) and FTLD ($N = 45$) patients (Rabinovici et al., 2011), and compared it to the performance of FDG-PET, which has an established diagnostic role in this scenario (Foster et al., 2007). PET scans were rated visually (blinded to clinical diagnosis) as PiB(+) or PiB(−) and as consistent with the FDG patterns of AD (temporoparietal-predominant hypometabolism) or FTLD (hypometabolism most severe in frontal or anterior temporal lobes). Scans were also classified quantitatively based on comparisons with normal controls. PiB visual reads were more sensitive for AD than FDG reads (89.5% vs. 77.5%) with similar specificity (83% vs. 84%). On quantitative classification, the sensitivity and specificity of PiB were essentially unchanged compared to visual reads, whereas FDG was slightly less sensitive (73%) but significantly more specific (98%). PiB outperformed FDG in a subset of 12 patients who underwent autopsy or carried a known pathogenic gene mutation, with an overall accuracy of 97% for PiB and 87% for FDG (see Section XIII for more details).

X. Dementia with Lewy Bodies and Parkinson's Disease

Dementia with Lewy bodies (DLB) is the second most common degenerative cause of dementia after AD (McKeith et al., 1996). Clinically, DLB is characterized by the coincident onset of cognitive decline (often affecting executive and visuospatial function with relative sparing of memory) and motor features of Parkinson's disease (PD) such as tremor, bradykinesia, rigidity, and postural instability (McKeith, 2006). Additional core features include visual hallucinations and fluctuations in cognition and arousal. DLB has significant clinical and pathological overlap with AD (McKeith, 2006). While pure DLB shows extensive deposition of α-synuclein protein in the form of Lewy bodies (Dickson, 2002), but no significant Aβ pathology, DLB with Aβ pathology (i.e., Lewy body variant of AD) is more frequently observed (Ballard et al., 2006). Evidence from *in vitro* binding and *in vivo* imaging studies suggests that PiB does not bind to α-synuclein deposits in detectable amounts (Bacskai et al., 2007; Burack et al., 2010; Fodero-Tavoletti et al., 2006; Klunk et al., 2003;), so PiB-PET can rule in or rule out the presence of significant Aβ pathology. Rowe et al. (2007) examined whether PiB retention can distinguish different types of dementia (AD, DLB, FTD), and found that cortical PiB retention was markedly elevated in every AD

subject regardless of clinical severity (n = 17) but was generally lower and more variable in DLB (n = 10) and below detection in FTD (n = 6). In the DLB subjects, high neocortical PiB retention (especially in precuneus/posterior cingulate) correlated with shorter time between the onset of cognitive impairment and clinical manifestation of DLB, suggesting that Aβ pathology may accelerate DLB development. Additionally, studies support that PiB can distinguish DLB from other neurodegenerative syndromes with similar clinical and pathological phenotypes, such as multiple systems atrophy (Claassen et al., 2011). When compared to Parkinson's disease dementia (PDD), another condition associated with extensive α-synuclein pathology, DLB subjects have significantly more Aβ deposition measured by PiB-PET (Claassen et al., 2011; Edison et al., 2008; Gomperts et al., 2008; Kalaitzakis et al., 2011; Maetzler et al., 2008), further supporting that Aβ deposition may have greater influence on the clinical development of DLB than PDD. However, vascular Aβ deposition is also common in PD and Aβ plaques are often found in PDD (Jellinger, 2003; Mastaglia et al., 2003). Johansson et al. (2007) reported that compared to HCs, cognitively intact PD patients do not show significantly increased PiB-PET retention, and PiB PET scan can be positive in more advanced PD patients. Indeed, higher PiB retention was reported in subjects with PDD (Foster et al., 2010; Kalaitzakis et al., 2011) and in two of three PiB-PET imaged PDD autopsy cases where PiB positivity was associated with the presence of frequent Aβ plaques (Fig. 5; Burack et al., 2010).

In conclusion, PiB imaging cannot distinguish DLB from AD given the high rate of Aβ co-pathology in DLB. This clinical distinction can be better accomplished by molecular imaging of the dopamine system, which is deficient in DLB but not AD (Koeppe et al., 2008). Further, a recent report has suggested that concomitant imaging of Aβ and markers of the presynaptic dopaminergic system in the same individuals aids in the differential diagnosis of DLB and AD (Villemagne et al., 2012). PiB imaging may have prognostic value, with a positive scan suggesting a more precipitous clinical course, though this needs to be more definitively demonstrated in longitudinal studies.

XI. Cerebral Amyloid Angiopathy (CAA)

An accumulating body of evidence from clinical, epidemiological, and autopsy studies suggest a relationship between cardiovascular disease (CVD) and Aβ pathology. Whether cerebral vascular pathology and Aβ deposition can influence each other, and the extent to which these changes affect cognition, is not clear. Recent autopsy studies and clinical imaging combining MRI and PiB-PET have contributed to our better understating of this potential relationship. CAA results from Aβ deposition in cerebral vessels' wall. Several postmortem studies reported high incidence of CAA (up to 98%) in

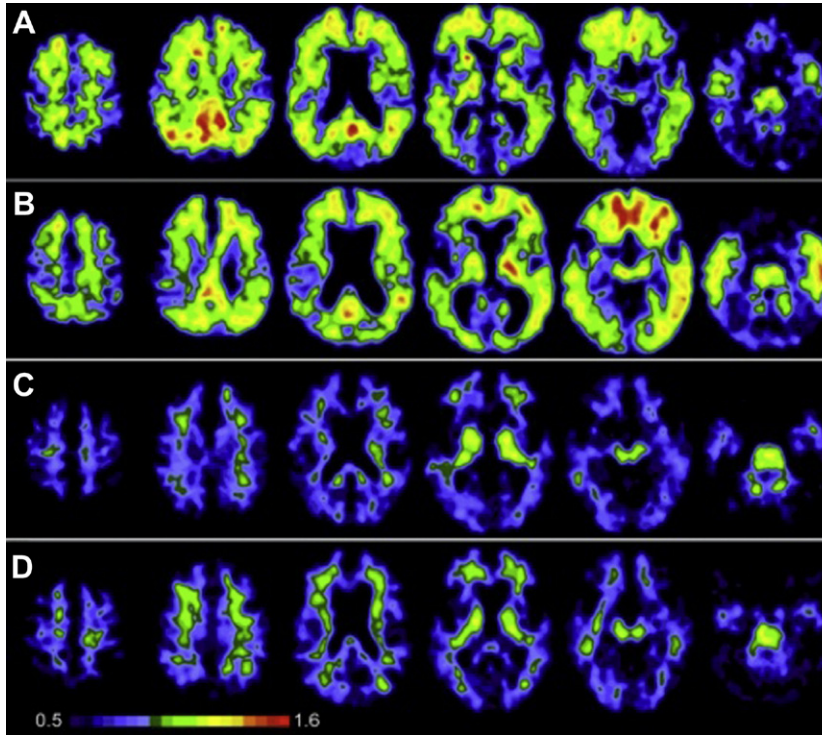

FIGURE 5 PiB-PET images from Parkinson's dementia cases with autopsy confirmed amyloid plaque pathology (A, B), a Parkinson's case without amyloid plaque pathology (C), and a control participant (D). For color version of this figure, the reader is referred to the online version of this book.
(from Burack et al., 2010).

AD (for review see (Jellinger, 2002)). While CAA can be found in the absence of dementia it is often found in association with AD. This is particularly the case in ApoE4 carriers, where CAA is associated with a risk of blood vessel rupture and cerebral hemorrhages including strokes (Maia et al., 2007) which can contribute to VaD. CAA-associated strokes are most frequently located in the occipital lobe (Attems et al., 2007; Rosand et al., 2005;) which is less severely affected with plaques when compared to frontal and parietal (precuneus) cortices. Both plaques and CAA are detectable with PiB (Bacskai et al., 2007; Ikonomovic et al., 2008; Lockhart et al., 2007) and contribute to PiB retention *in vivo*.

Johnson et al. (2007) evaluated the sensitivity of PiB-PET to detect CAA in six nondemented subjects diagnosed with clinically probable CAA and compared them to patients with probable AD, and HCs. They found that all of the CAA and AD subjects were PiB(+). Global cortical PiB retention in the CAA group was significantly higher relative to HC subjects but was

lower than in AD subjects. The occipital-to-global PiB ratio, however, was significantly greater in CAA than in AD subjects—consistent with the known predilection of CAA for the occipital lobe. Similarly, in a 42-year-old man with Iowa-type hereditary CAA, PiB retention was observed only in the occipital cortex, consistent with the pathology of this type of CAA (Greenberg et al., 2008). These findings have been replicated in additional CAA cohorts showing significantly increased occipital-to-global ratio of PiB retention (Ly et al., 2010).

XII. Atypical Presentations of AD

While episodic memory loss is considered the clinical hallmark of AD, ~15% of AD patients seen at academic centers have a nonamnestic presentation (Snowden et al., 2007). Two clinical syndromes in particular—posterior cortical atrophy (PCA), a progressive disorder of visuospatial function, and logopenic-variant primary progressive aphasia (lvPPA), a language disorder characterized by difficulties with naming, word retrieval, and repetition—have been strongly associated with AD pathophysiology (Alladi et al., 2007; Gorno-Tempini et al., 2004; Mesulam et al., 2008; Tang-Wai et al., 2004). These nonamnestic presentations have been incorporated into new AD diagnostic guidelines (McKhann et al., 2011).

Amyloid PET can be helpful in diagnosing AD in patients presenting with PCA and lvPPA during life, particularly since these syndromes are associated with early age-of-onset, and the alternative causative pathologies fall in the FTLD (non-Aβ) family. Indeed, a number of studies have demonstrated that patients diagnosed with PCA and lvPPA at expert centers are nearly always PiB(+) (de Souza et al., 2011; Formaglio et al., 2011; Leyton et al., 2011; Ng et al., 2007a; Rabinovici et al., 2008; Rabinovici et al., 2011; Rosenbloom et al., 2011). PiB may also be useful in diagnosing AD in patients with a dysexecutive-behavioral presentation (frontal-variant AD) (Johnson et al., 1999) and corticobasal syndrome, a disorder of sensorimotor integration, primary motor, and cognitive function (Lee et al., 2011), though data on these syndromes are limited to case reports (Laforce & Rabinovici, 2011). Interestingly, most group-level analyses have found that the distribution of amyloid in PCA and lvPPA is similar to the distribution in AD, though neurodegeneration patterns (as determined by MRI and FDG-PET) are distinct, with more occipital involvement in PCA and asymmetric left hemisphere degeneration in lvPPA (de Souza et al., 2011; Leyton et al., 2011; Rabinovici et al., 2008; Rosenbloom et al., 2011) (Fig. 6). These findings, along with the discordance between PiB and atrophy/hypometabolism patterns in typical AD ((Rabinovici et al., 2010), also see sections below, Section XIV), suggest that the burden and spatial distribution of fibrillar Aβ (as imaged by PiB) do not explain the clinical and anatomic heterogeneity of AD.

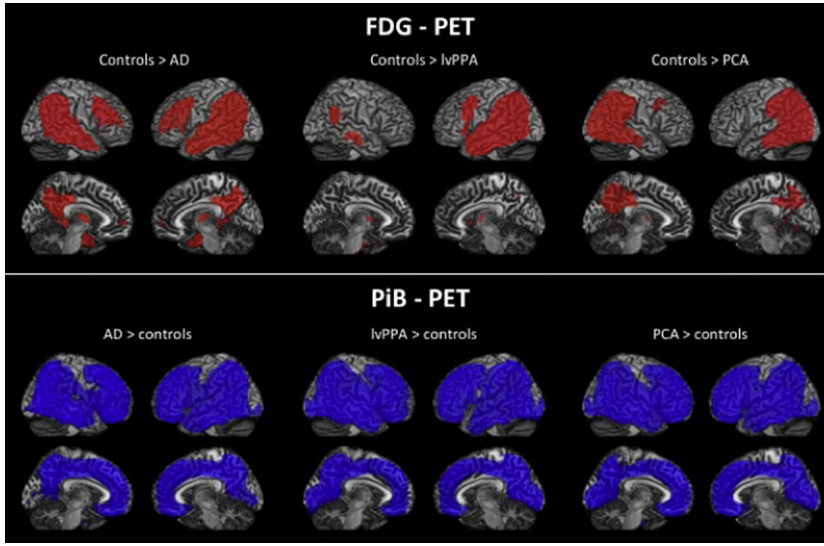

FIGURE 6 Patterns of FDG and PIB binding in amnestic (AD), language (lvPPA) and visual (PCA) variants of AD compared with HCs. Shown are t-maps after correction for multiple comparisons (family-wise error correction at $p < 0.05$). Red in the FDG maps indicates significantly more hypometabolism in the patient groups compared with controls, whereas blue in the PIB maps indicates significantly more amyloid deposition in the patient groups. FDG patterns are distinct and correlate with the clinical deficits, while PIB binding is diffuse and indistinguishable across variants. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

XIII. Postmortem Validation of PiB-PET Imaging

From the earliest *in vivo* PiB-PET imaging studies it has been suggested that PiB retention reflects the extent of Aβ pathology in the brain (Klunk et al., 2004). However, strong, direct evidence in support of this idea became available only recently, after some of the PiB-PET imaged subjects came to autopsy. Autopsy studies of PiB-PET imaged brains allowed for the first time, that correlations can be examined between antemortem PiB retention levels and region-matched postmortem measures of fibrillar Aβ load and other neuropathology in the same brains. To date there has been more than a dozen of PiB-PET autopsy case reports in the literature (see Table I) that will facilitate elucidating the pathological substrates of PiB retention in brains of cognitively normal aged people and subjects with AD or other dementias.

The first PiB-PET autopsy case was described in 2007 by Bacskai and colleagues (Bacskai et al., 2007). This subject had a clinical diagnosis of DLB with mild impairment on the clinical dementia rating (CDR = 1) and mini-mental state examination (MMSE = 25) scales. A PiB-PET scan was performed 3 months prior to autopsy, and it showed positive PET

TABLE I Overview of Studies Reporting PiB-PET Autopsy Cases

Reference	[a] *PiB (+/–)*	[b] *Clinical diagnosis (at time of PET scan)*	*Cognitive score (at time of PET scan)*	*PET-to-death interval (months)*	[c] *Cerebral amyloid angiopathy (severity)*	[c] *Cortical NP frequency*	[c] *Cortical DP frequency (load)*	[d] *CERAD/ NIA-RI diagnosis of AD*	*Braak stage for NFT*
[1]	+	DLB	CDR = 1/ MMSE = 25	3	Severe	Sparse	Frequent	Possible/IL	IV
[2]	+	AD	MMSE = 1	10	Sparse	Frequent	Frequent	Definite/HL	VI
[3]	–	Normal	CDR = 0	30	Mild	Sparse	Focally frequent	Possible/LL	III
[4]	–	CJD	n/s	<1	present (n/s)	None	None	n/s	n/s
[4]	–	CJD	n/s	<1	present (n/s)	None	Sparse	n/s	n/s
[5]	+	PDD	CDR = 2/ MMSE = 23	< 15	Mild	Sparse	Frequent	Possible/LL	III
[5]	+	PDD	CDR = 2/ MMSE = 11	<15	None	Sparse	Frequent	Possible/LL	III
[5]	–	PDD	CDR = 1/ MMSE = 24	<15	None	None	Sparse	n/s	I
[6]	+	DLB	MMSE = 10	18	Mild	Moderate	Frequent	n/s/LL	III
[7]	+	AD	MMSE = 5	35	present (n/s)	Frequent	Frequent	Definite/ HL	VI
[8]	+	Normal	CDR = 0	16	present (n/s)	Sparse	High (>5%)	Normal/NO	IV
[8]	+	Dementia	CDR = 1	2	present (n/s)	Moderate	High (>5%)	Probable/IL	III
[8]	–	Normal	CDR = 0	20	None	None	Low (<5%)	Normal/NO	IV
[8]	–	Normal	CDR = 0	28	None	Moderate	Low (<5%)	Possible/NO	III
[8]	–	Normal	CDR = 0	28	None	Moderate	Low (<5%)	Possible/NO	IV
[8]	–	MCI	CDR = 0.5	13	present (n/s)	Moderate	Low (<5%)	Possible/IL	III
[9]	–	DLB	MMSE = 10	17	Moderate	Focally frequent	Focally frequent	[e]Definite/LL	II

[a] PiB positivity (+) is defined by either local cutoffs defined by the authors or by cutoffs in standard use such as a DVR>1.4 (or BP>0.4) or an SUVR>1.5

[b] Clinical diagnosis, AD (Alzheimer disease), CJD (Creutzfeldt–Jakob disease), DLB (dementia with Lewy bodies), MCI (mild cognitive impairment), PDD (Parkinson disease dementia). Highest regional values are shown for congophilic amyloid angiopathy and frequencies of neuritic plaques (NP) and diffuse plaques (DP)

[c] CERAD = Consortium to establish a registry for Alzheimer's disease (diagnoses of possible, probable, or definite AD); NIA-RI = The National Institute on Aging and Reagan Institute (LL = low likelihood of AD, IL = intermediate likelihood of AD, HL = high likelihood of AD, NO = not AD)

[d] Diagnosis of definite AD was based on a single area of frequent neuritic plaques in the frontal cortex and strict application of the CERAD criteria.

NFT = neurofibrillary tangles

n/s = not specified

Modified from Ikonomovic et al. (2012).

retention when assessed using the reference Logan graphical analysis (Logan et al., 1996), with distribution volume ratios (DVR) ranging between 1.30 in the parietal cortex and 1.50 in the cingulate cortex. Postmortem neuropathology evaluation of the neocortex detected Lewy bodies in temporal and cingulate cortices, and moderate NFT in temporal, parietal, and occipital cortices, consistent with Braak stage IV (Braak & Braak, 1991). However, Aβ plaque pathology was surprisingly low in this case, with only rare neocortical cored plaques and numerous diffuse plaques observed using Aβ immunohistochemistry (6F/3D antibody). The low frequency of neuritic plaques and the NFT pathology in this case resulted in diagnosis of "possible AD" based on the Consortium to Establish a Registry of Alzheimer's Disease (CERAD) (Mirra et al., 1991) and in an "intermediate likelihood of AD" based on the National Institute on Aging-Reagan Institute (NIA-RI) criteria (Consensus, 1997). Interestingly, both Aβ immunohistochemistry and PiB fluorescence in tissue sections revealed severe CAA. Biochemical analyses of soluble and insoluble Aβ concentrations in this PiB-PET positive case showed a preponderance of Aβ40 over Aβ42, supporting that vascular amyloid was the dominant form of Aβ pathology.

Several postmortem studies of AD cases without PiB-PET scan confirmed that CAA is a major pathologic substrate for PiB retention in the brain, and provided additional valuable information regarding PiB retention in dementia cases (see Section XI). Ikonomovic and colleagues (Ikonomovic et al., 2008) performed histological characterization of PiB retention using 6-CN-PiB, a highly fluorescent derivative of PiB, on postmortem tissue sections from multiple brain regions in 27 dementia cases from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC). PiB retention was most prominent in neocortical Aβ immunoreactive (6E10, 10D5, Aβ40, and Aβ42 antibodies) deposits in cerebral vasculature and in classic cored and neuritic plaques. PiB retention to neocortical and striatal diffuse plaques was far less prominent but still detectable, while diffuse Aβ plaques in the cerebellum were not detectable using 6-CN-PiB (Ikonomovic et al., 2008). A similar observation of PiB binding to CAA and classical and diffuse plaques was reported using [H-3]PiB autoradiography on brain tissue sections (Lockhart et al., 2007; Thompson et al., 2009). Lockhart and colleagues also reported that PiB binds to NFT (Lockhart et al., 2007); however, other studies did not support this idea and instead suggested that the extracellular ("ghost") type of NFT is more likely to bind PiB due to the presence of Aβ fibrils in these extracellular tau aggregates (Ikonomovic et al., 2008; Fig. 7). Figure 7 illustrates selectivity of PiB retention to Aβ deposits in postmortem brain tissue sections; there is a very good correspondence between PiB retention and Aβ plaques while no binding of PiB to intracellular NFT is detectable. Regardless, at doses of PiB used for PET imaging it is unlikely that NFT could be detected *in vivo*.

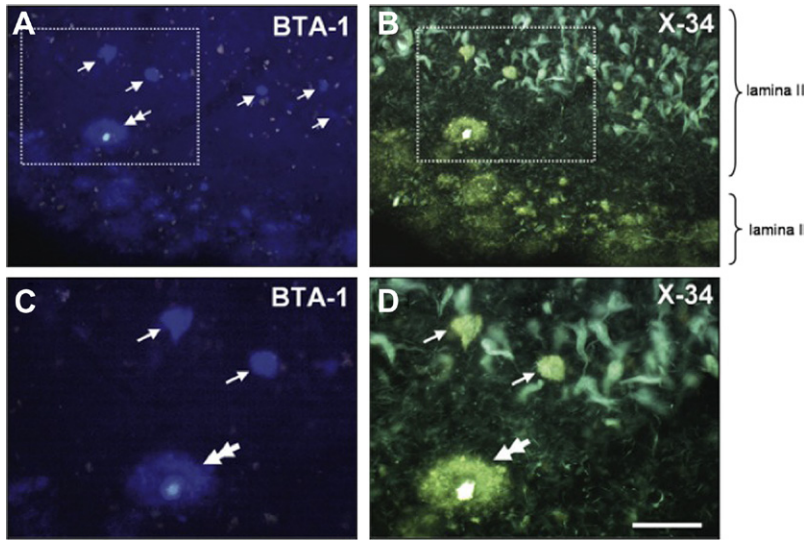

FIGURE 7 Double-histofluorescence staining of a single section of an AD entorhinal cortex, using BTA-1 (1 μM, A and C; U filter) and X-34 (100 μM, B and D; V filter) histofluorescence. Tissue autofluorescence (lipofuscin) is seen as bright bleed-through signal in A and C. Boxed areas in A and B delineate areas of higher magnification in C and D, respectively. Subpial diffuse Aβ plaques, and a single cored plaque (C and D, double-arrows) in lamina I are seen with both compounds. In lamina II, BTA-1 labels only a few isolated structures (A, arrows), while X-34 also labels abundant NFTs and neuropil threads (B). BTA-1 labeled structures inside layer II islands (C, arrows) with X-34 histofluorescence (D, arrows), similar to the neighboring cored plaque (D, double arrow); this makes them distinct from the surrounding NFTs that are seen with X-34 histofluorescence (B, D). Scale bar = 100 μm (A, B), 50 μm (C, D). For color version of this figure, the reader is referred to the online version of this book.
(from Ikonomovic et al. 2008)

It has also been of interest to determine if PiB retention reflects other types of intracellular protein aggregates such as α-synuclein in Lewy bodies (LB). Using [H-3]PiB binding, it was observed that PiB has very low binding affinity for α-synuclein fibrils, and no binding was detected in homogenates of DLB brains free of Aβ deposits (Fodero-Tavoletti et al., 2007). Collectively, these postmortem findings support that PiB retention is highly specific for fibrillar Aβ deposits, while binding to other types of neuropathology is negligible. The strong binding of PiB to CAA and classic cored plaques is due most likely to dense β-sheet structure of Aβ fibrils in these lesions. In contrast, it has been assumed that diffuse plaques lack fibrillar structure and therefore cannot bind PiB. The presence of detectable 6-CN-PiB histofluorescence (Ikonomovic, et al., 2008) and [H-3]PiB autoradiography signal (Lockhart et al. 2007) in diffuse plaques support that these lesions can retain PiB *in vivo* as a result of fibrillar Aβ present even in diffuse plaques. This is in agreement with reports of high PiB retention levels in familial AD

(presenilin mutation) and variant AD subjects with large amounts of diffuse striatal plaques and cortical cotton wool plaques (Klunk et al., 2007; Koivunen et al., 2008). The absence of postmortem PiB labeling of diffuse plaques in the cerebellum (Ikonomovic et al., 2008) justifies using this region as a reference area for *in vivo* PiB retention analyses (Lopresti et al., 2005).

The first correlation analysis of region-matched antemortem PiB retention and postmortem measures of neuropathology was reported in a PiB-PET imaged typical AD subject with end-stage disease (Ikonomovic et al., 2008). The 64-year-old female subject examined in that study had a clinical diagnosis of probable AD and a positive PiB-PET scan 10 months prior to death. PiB retention was positive in all cortical regions (DVR range 1.59–2.38). Neuropathological diagnosis was "definite AD" by the CERAD criteria (Mirra et al., 1991) and Braak stage was V/VI (Braak & Braak, 1991). Frequent cortical plaques and mild CAA were Aβ immunoreactive (6E10 antibody) and positive for 6-CN-PiB fluorescence. Both Aβ immunoreactive and 6-CN-PiB positive plaque loads (% area) correlated strongly with region-matched DVR values determined antemortem in the same subject (Ikonomovic et al., 2008). Strong direct correlations were also observed between antemortem DVR values and region matched postmortem biochemistry measures of total Aβ42 and Aβ40 concentration or [H-3]PiB binding in frozen tissue homogenates from this case. Similar findings were reported by Kadir and colleagues (2011) who examined another case of typical end-stage AD; this 61-year-old female with severe dementia (MMSE = 5) was the first patient ever imaged using PiB-PET. She underwent PiB-PET imaging 35 months prior to death, and there was strong PiB-PET positivity in all regions examined. Neuropathology findings included frequent or widespread Aβ plaques detected using a battery of different Aβ antibodies (6E10, 4G8, 6F/3D, Aβ40, and Aβ42), neuropathology diagnosis was "definite AD" by the CERAD criteria (Mirra et al., 1991) and Braak stage for NFT was V/VI. Strong, direct correlations were detected between antemortem standardized uptake values (SUVs) and region-matched measures of Aβ plaque distribution, Aβ concentration, and [H-3]PiB binding (Kadir et al., 2011). Collectively, the results of these two studies of PiB autopsy brains from typical end-stage AD patients provide further support that *in vivo* PiB-PET retention reflects fibrillar Aβ burden. Other PiB brain autopsy studies examined additional cases with antemortem clinical diagnosis of DLB (Kantarci et al., 2010; Ikonomovic et al., 2012) and cases with PDD (Burack et al., 2010). These studies led to the conclusion that in patients with concomitant LB and Aβ pathology, it is the fibrillar Aβ burden, and not LB, which determines PiB retention *in vivo* (see Table 1).

The presence of even minimal Aβ deposits in a subject with a negative PiB-PET scan brings into question the sensitivity of this technique. Several postmortem studies reported various amounts of Aβ pathology in brains of PiB(–) subjects. Cairns et al. (2009) reported autopsy findings in a

91-year-old subject who had a negative PiB–PET scan (neocortical PiB retention ranged from 0.03 to 0.19) and normal cognition (CDR = 0) when evaluated 30 months prior to death. The subject later developed very mild dementia (CDR = 0.5) and underwent CSF analysis for Aβ/tau. Based on the neuropathology evaluation the case was diagnosed as "possible AD" by the CERAD criteria (Mirra et al., 1991) with a low likelihood that the mild dementia was caused by AD, based on the NIA-RI criteria (Consensus, 1997). There were sparse to focally frequent diffuse plaques, infrequent neuritic plaques, and mild CAA. Up to 5.4% area of neocortex was covered with Aβ immunoreactive plaques (10D5 antibody), an Aβ1-42 ELISA detected high levels of Aβ1-42 in cortical areas (range 687–1785 pmol/g wet tissue), and cortical [H-3]PiB binding ranged between 116–295 pmol/g. Interestingly, CSF was sampled 1 year after the PiB-PET scan was done, ~18 months prior to death, and it showed abnormal Aβ/tau levels, leading Cairns and colleagues to suggest that CSF profiling is more sensitive than PiB-PET in detecting fibrillar Aβ deposits in the brain (Cairns et al., 2009).

Ikonomovic et al (2012) reported autopsy findings in a PiB(–) subject with antemortem diagnoses of DLB and possible AD. PiB retention was low (DVR<1.2 in all cortical regions); however, postmortem neuropathology analysis 17 months later revealed mild to moderate and even focally frequent neocortical neuritic plaques which allowed for a diagnosis of "definite AD" by strict CERAD criteria (Mirra et al., 1991). Aβ immunoreactive plaque load was up to 1.8% of total plaque load but the majority of plaques were diffuse and they labeled weakly with PiB. While cortical Aβ1-40 concentration levels (up to 233 pmol/g) were similar to those in a typical PiB(+) AD case (Ikonomovic et al., 2008), Aβ1-42 concentrations were lower in all brain areas except the frontal cortex, where values (788 pmol/g) approached those measured in a typical PiB(+) AD case. However, [H-3]PiB binding in the frontal cortex and all other cortical regions from the PiB(–) case was low (60 pmol/g or less). The low ratios of PiB retention to Aβ measures in both histological and biochemical assays indicated very low fibrillar Aβ load in this PiB(–) brain (Ikonomovic et al., 2012). It is interesting that the amount of neuritic plaque pathology in this case was more substantial than in the PiB(–) case reported by Cairns et al. (Cairns et al., 2009), where "definite AD" diagnosis could result only by applying Khachaturian neuropathologic criteria (Khachaturian, 1985). Both cases were analyzed using the same methodology; however, the Cairns' PiB(–) case had greater cortical Aβ-immunoreactive plaque load (up to 5.4 % area), Aβ1-42 concentration (687–1785 pmol/g wet tissue), and [H-3]PiB binding (116–295 pmol/g). The longer PET-to-death interval in the Cairns case (30 months) compared to the Ikonomovic case (17 months) may explain these differences. PiB(–) scans were also reported in two autopsy cases with a diagnosis of CJD (Villemagne et al., 2008) and in four autopsy cases with either mild (CDR = 0.5) or no cognitive impairment (Sojkova et al., 2011). While CJD cases in the

study by Villemagne and colleagues had either absent or minimal Aβ plaques (Villemagne et al., 2008), several [C-11]PiB negative subjects examined by Sojkova and colleagues had moderate numbers of neocortical neuritic plaques (Sojkova et al., 2011).

The sensitivity of PiB-PET imaging is not well understood, and this technique may not be 100% sensitive for the presence of histologically detectable Aβ even if it were determined close to the time of the *in vivo* scan. On the other hand, so far there has been no report of an *in vivo* PiB(+) subject who failed to show Aβ deposits at autopsy, supporting good specificity of this technique. To-date, the most likely explanation for the few *in vivo* PiB(−) cases that have detectable postmortem Aβ is a combination of the following: (1) low amounts of Aβ that are below the *in vivo* threshold of the PiB-PET imaging technology and (2) a high percentage of nonfibrillar deposits of Aβ which are not easily detected with PiB-PET. There is some evidence that Aβ42 is more closely associated with *in vivo* PiB retention than Aβ40 (Ikonomovic et al., 2008, 2012). Additional analyses of large numbers of PiB(−) and PiB(+) cases, with short imaging-to-autopsy interval, are required to establish a threshold level of Aβ pathology necessary for *in vivo* PiB-PET detection.

XIV. Amyloid Imaging Compared to Other Biomarkers

A. PiB and FDG

Decreases in cerebral glucose metabolism, measured by FDG, show a characteristic regional pattern of posterior temporoparietal > frontal hypometabolism in AD (Foster et al., 2007; Friedland et al., 1983;Herholz, Carter, & Jones, 2007; Jagust et al., 2007). Similar changes have been reported in cognitively normal individuals at high risk for AD due to expression of the Apo-E4 alelle (Reiman et al., 1996; Small et al., 2000). Changes in cerebral metabolism also have been detected in MCI in many studies (Arnáiz et al., 2001; Chetelat et al., 2003a; Chetelat et al., 2003b; Del Sole et al., 2008; Garibotto et al., 2008; Li et al., 2008; Mevel et al., 2007; Mosconi et al., 2006; Mosconi et al., 2008 Perneczky et al., 2007). These early changes suggest FDG could play a predictive role in detecting which normal controls or MCI patients are most likely to convert to AD (Yuan et al., 2008). Indeed, several studies have shown that abnormalities in FDG PET predict progression from MCI to AD (Anchisi et al., 2005; Drzezga et al., 2005; Mosconi et al., 2004).

In the initial PiB-PET study, the largest and only significant difference in glucose metabolism (determined with FDG PET) between AD patients and control subjects was observed in parietal cortex. An inverse correlation between PiB retention and glucose metabolism was observed in most cortical areas, but this trend reached significance only in the parietal cortex. The lack of correlation between PiB and glucose metabolism in the frontal

cortex suggests that Aβ deposition is not sufficient to *locally* reduce cerebral metabolism, suggesting that perhaps compensatory changes in neurotransmitter systems (i.e., DeKosky et al., 2002; Ikonomovic et al., 2007) in the frontal cortex delay FDG hypometabolism in frontal brain regions. Edison et al. (2006) investigated the association between PiB and FDG PET in AD. AD subjects showed significant increases in PiB retention in cingulate, frontal, temporal, parietal, and occipital cortical areas and levels of temporal and parietal rCMRglc were reduced by 20% in AD. Higher PiB retention correlated with lower rCMRglc in temporal and parietal cortices, but not in frontal areas. While these typical negative correlations were observed in AD, subjects with MCI often display positive correlations between PiB and FDG, reflecting increased brain reserve in those subjects who remain at the MCI level of cognitive impairment further into the process of Aβ deposition (Cohen et al., 2009).

Forsberg et al. explored MCI subjects with PiB and FDG PET, as well as assessment of cognitive function and CSF sampling. The MCI subjects that later converted to AD showed significantly higher PiB retention compared to nonconverting MCI patients. However, there was no significant difference in rCMRglc between MCI patients and HCs in any cortical brain region, suggesting PiB may better predict clinical conversion than FDG-PET. However, Furst et al. (2010) demonstrated that cognitive performance in AD correlated strongly with FDG but not at all with PiB, and did not demonstrate any significant correlations between PIB and FDG

Ng et al. (2007) compared a visual assessment to a quantitative assessment of PiB and FDG PET data for detection of AD compared to cognitively intact controls. Visual agreement between readers was excellent for PiB (kappa = 0.90) and good for FDG (kappa = 0.56). Based on the clinical diagnosis, Ng et al. found PiB was more accurate than FDG both on visual reading (accuracy, 90% vs. 70%) and ROC analysis (95% vs. 83%). The authors concluded that the visual analysis of PiB images appears more accurate than visual reading of FDG for identification of AD and had accuracy similar to quantitative analysis of a 90 min dynamic scan. Similar results were found in the Rabinovici et al. (2011) differential diagnosis study described above; inter-rater agreement was significantly higher for PiB (kappa = 0.96) than FDG (kappa = 0.72), as was agreement between visual and quantitative classifications (average kappa = 0.90 for PiB, 0.66 for FDG). The authors concluded that PiB was the superior qualitative technique in that visual assessment was both more accurate and more precise. While PiB and FDG demonstrate high (94%) agreement in differentiating AD from normal controls, agreement is lower in classifying MCI subjects (54%) (Li et al., 2008). Li et al. argues that "combining the two modalities improves the diagnostic accuracy for MCI." In addition, when exploring the use of PiB and FDG among both AD and MCI subtypes it was demonstrated that while PiB and FDG displayed similar diagnostic accuracy, PiB was

significantly better at separating MCI subtypes (Lowe et al., 2009). These findings are not surprising since the two tracers provide complementary information, with PiB quantifying molecular pathology, and FDG demonstrating neuronal dysfunction. The complementary nature of the two techniques are reflected in the new diagnostic guidelines for MCI and AD dementia, which require biomarker evidence of both Aβ deposition (CSF or amyloid PET) and neurodegeneration (hypometabolism on FDG-PET or atrophy on MRI) to diagnose AD pathophysiology with high-likelihood during life (McKhann AD criteria, Albert MCI criteria).

B. PiB and MRI

Many studies have demonstrated hippocampal atrophy in AD and MCI (Apostolova et al., 2006a; Becker et al., 2006; Grundman et al., 2002; Moretti et al., 2007; Morra et al., 2009). Furthermore, several studies have shown that the rate of hippocampal atrophy may identify those MCI patients soon to convert to clinical AD (Apostolova et al., 2006b; Apostolova et al., 2008; Chetelat et al., 2008; de Toledo-Morrell et al., 2004; Devanand et al., 2007; Grundman et al., 2002; Jack et al., 1999; Jack et al., 2000; van de Pol et al., 2007; Wang et al., 2009). When PiB-PET was correlated with volumetric MRI measurements in AD, a significant, positive correlation was observed between rates of whole brain atrophy and cortical PiB retention (Archer et al., 2006; Chetelat et al., 2010; Fotenos et al., 2008; Frisoni et al., 2009). In one study, PiB retention was shown to predict later decline in brain volume (Scheinin et al., 2009). However, in cognitively normal elderly, volume decline in the decade preceding PiB-PET is not correlated with cortical PiB retention (Driscoll et al., 2010). However, Chetelat et al. (2012), recently showed that cognitively unimpaired PiB(+) individuals have significantly higher rates of brain atrophy than their PiB(–) counterparts. Further, Jack et al. (2009) explored PiB and MRI across the AD continuum and observed a significant correlation between MMSE and ventricular atrophy, with only a weak correlation between PiB and ventricular size, suggesting a complementary use of PiB-PET and MRI in detection of MCI and AD, as reflected in the new diagnostic criteria (Jack et al., 2011).

C. PiB and Cerebrospinal Fluid (CSF) Aβ

Because neuritic Aβ plaques and NFT do not develop simultaneously in the brain, the availability of lesion-specific radioligands would facilitate evaluations of AD pathology *in vivo*. Histopathology studies demonstrated that PiB retention is specific for fibrillar Aβ pathology and that PiB binds negligibly or not at all to NFT and Lewy bodies (Fodero-Tavoletti et al., 2007; Ikonomovic et al., 2008; Lockhart et al., 2007; Thompson et al., 2009). Besides 2-(1-{6-[(2-[F-18] fluoroethyl) (methyl)amino]-2-naphthyl}

ethylidene)malononitrile (FDDNP) PET which has been claimed to detect both Aβ plaques and NFT (Small et al., 2006), and some emerging tau-binding candidate radioligands such as [F-18]THK523 (Fodero-Tavoletti et al., 2011), none of the currently used imaging radiotracers allows for measurements of aggregated tau or phosphorylated tau (p-tau) pathology in brain tissues in living patients. Cerebrospinal fluid (CSF) analysis of Aβ42 and p-tau concentrations is an alternative, indirect method for quantifying both types of pathology in the brain; it has been reported to have high accuracy for identifying individuals with incipient AD (Mattsson et al., 2009) and for predicting the development and rate of cognitive decline (Buchhave et al., 2012; Fagan et al., 2007; Snider et al., 2009). CSF from AD patients contains higher concentrations of total and phosphorylated tau and lower levels of Aβ42 which correlate with the presence of post-mortem neurofibriallary and amyloid pathology respectively (Strozyk et al., 2003). However, the exact relationship between the amounts of fibrillar Aβ in brain parenchyma and soluble Aβ concentration in CSF is unclear. Based on a study in Tg2576 mice (Kawarabayashi et al., 2001) it has been assumed that lower CSF Aβ42 reflects deposition of fibrillar Aβ in brain tissues; however, no direct evidence from human studies is available to confirm this hypothesis and alternate hypotheses for lowered CSF Aβ such as impairments in clearance may apply better in humans.

Several clinical studies examined the relationship between Aβ changes in the brain and CSF by measuring *in vivo* PiB-PET retention and CSF Aβ42 concentration in the same subjects. A strong inverse correlation was observed between the two biomarkers, both in a mixed cohort of cognitively normal and demented subjects (Fagan et al., 2006) and in a homogeneous population of cognitively intact individuals (Fagan et al., 2009). While these associations were initially modeled as linear correlations, it has become increasingly recognized that the relationship between PiB retention and CSF Aβ42 is better modeled by a nonlinear approach. As expected, there was no correlation between PiB retention and CSF tau levels (Fagan et al., 2006). Similar associations between amyloid imaging and CSF Aβ were observed in cohorts of cognitively healthy (Storandt et al., 2012), MCI (Forsberg et al., 2007; Koivunen et al., 2008), and AD subjects (Grimmer et al., 2009). In a longitudinal study by Forsberg et al. (2007), all MCI subjects that converted to AD had high PiB retention, but <50% had pathological levels of Aβ42 in the CSF, suggesting that amyloid imaging may be more sensitive than CSF Aβ42 concentration in identifying MCI subjects who will develop AD (Forsberg et al., 2007). Observations by Koivunen et al. (Koivunen et al., 2008) lent further support to this idea; high PiB retention was detected in 87% of MCI patients while only 53% of MCI subjects had pathological levels of CSF Aβ42. The reason why some PiB(+) MCI subjects have normal Aβ42 concentration in the CSF is unknown. Grimmer and colleagues also reported an inverse correlation between overall [C-11]PiB retention in the brain and

CSF Aβ42 levels in their cohort of AD subjects (Grimmer et al., 2009)—particularly in paraventricular regions, and more recently the same group reported that BACE1 activity in the CSF correlates with PiB-PET retention levels in the parahippocampal gyrus, thalamus, and pons (Grimmer et al., 2012).

In a cohort representing an entire spectrum of cognitive decline, Tolboom and colleagues (Tolboom et al., 2009a) compared CSF biomarkers to both PiB and [F-18]FDDNP. After adjusting for potential confounding variables, increased global or regional PiB retention was associated with low CSF Aβ42 (Tolboom et al., 2009). No association was observed between PiB and CSF tau, in agreement with some (Fagan et al., 2006; Forsberg et al., 2008) but not other (Storand et al., 2012) studies. Collectively, these studies support that PiB retention specifically reflects Aβ plaque pathology in the brain. In contrast, high [F-18]FDDNP retention was associated with high CSF tau, but no correlation was found with CSF Aβ42, suggesting that this radiotracer is more associated with NFT pathology in AD brains (Tolboom et al., 2009).

Cairns and colleagues studied a cognitively normal subject (CDR = 0) who had a negative PiB-PET scan; however, 12 months after the PET scan CSF analysis showed decreased Aβ42 and slightly increased tau and p-tau concentration, 18 months after the PET scan there were clinical signs of a very mild dementia (CDR = 0.5), and 30 months after the PET scan the subject died and neuropathology examination found evidence of primarily diffuse neocortical Aβ plaques (NIA-RI low likelihood AD) (Cairns et al., 2009). These observations may suggest that CSF Aβ42 may be a more sensitive biomarker for detection of AD pathology when compared to PiB-PET. Additional studies in large numbers of subjects are needed to determine if amyloid imaging of fibrillar Aβ load or CSF Aβ concentration is a more sensitive biomarker and which one is better at predicting progression from MCI to AD.

C. PiB and Neuroinflammation

It is well known that inflammatory processes contribute to pathogenesis of AD. Activation of microglia appears to be an early reactive mechanism in response to amyloid deposition, and brain inflammation may even precede amyloid plaques and tangles in AD brain (for review see McGeer & McGeer, 2010). Studies in transgenic mice demonstrated that anti-inflammatory therapies are capable of reducing both microglia/cytokine reaction and Aβ load as determined by percent area and ELISA measurements (Lim et al., 2000). Thus, PET imaging of activated microglia, using radioligands that can specifically bind to peripheral benzodiazepine receptors expressed by these cells, is a valuable tool for evaluating the extent of inflammatory processes in living patients with chronic neurodegenerative disorders including AD (Venneti et al., 2009).

Several *in vivo* imaging studies examined both amyloid deposition and microglial activation using PET. Wiley and colleagues (2009) examined potential associations between amyloid pathology and microglial activation using PiB and (R)-PK11195 ([1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide], a PET radiotracer for imaging peripheral benzodiazepine binding sites), respectively, in six mild-moderate AD, six MCI, and five cognitively normal subjects. There was no association between increased (R)-PK11195 uptake and positive PiB PET retention, suggesting that microglia activation occurs only during specific stages of amyloid deposition, and (R)-PK11195 may lack sensitivity to detect such changes. Similarly, in a study of amnestic MCI, Okello and colleagues (2009) found that not all of their PiB positive subjects had increased uptake of (R)-PK11195. Therefore, using this specific radioligand for measuring activated microglia *in vivo*, inflammatory process can be detected only in a subset of patients with increased amyloid burden. The same group examined 13 AD subjects and reported concomitant increases in (R)-PK11195 and PiB signal in multiple brain areas from AD brains. Interestingly, increased [C-11](R)-PK11195 uptake, but not PiB retention, correlated with impaired cognition in this AD cohort (Edison et al., 2008).

Collectively, these studies indicate that imaging brain inflammation is a valuable approach in evaluating AD pathology *in vivo*; however, more sensitive radioligands need to be developed. Furney et al., (2011) reported that compared to *in vivo* brain structural imaging alone, a combination of MRI imaging and inflammation (cytokine) biomarkers is a better predictor of a conversion from MCI to AD. PiB-PET imaging is particularly useful for monitoring changes in amyloid load in response to anti-amyloid therapies. While Aβ immunization appears to be effective in reducing amyloid pathology in AD patients, this intervention has been observed to activate microglia reaction in the brain and it can result in severe side effects (Boche et al., 2010). Therefore, combining *in vivo* PiB-PET imaging with biomarkers of inflammation will be of a particular importance when evaluation AD patients undergoing such therapies.

XV. Amyloid Imaging in AD Drug Development

Amyloid imaging will likely have two complimentary roles in clinical trials of future AD therapies. At the level of subject selection, amyloid PET will help ensure that patients enrolled in AD treatment trials truly have underlying Aβ deposits. This should increase the efficiency of AD- specific trials at the MCI phase (by eliminating the 25–40% of patients with non-AD causes of MCI who are unlikely to respond to the biological intervention) (Lorenzi et al., 2010), and ultimately by enabling primary prevention trials at the preclinical stage (Bateman et al., 2011; Reiman et al., 2011). Second, amyloid

PET may be useful for demonstrating a biological effect of anti-Aβ therapies in early stages of drug development. Two studies thus far have illustrated this potential application of amyloid imaging. In a phase 2 trial of bapineuzumab, a humanized monoclonal antibody targeting Aβ, 19 patients receiving active treatment, and 8 receiving placebo underwent PiB-PET at baseline and following 18 months of treatment (up to six infusions) (Rinne et al., 2010). Mean cortical PiB SUVr values increased by an average of 16.9% from baseline in the placebo group, but *decreased* by an average of 8.5% from baseline in the active treatment group, resulting in an observed treatment effect of ~25%. Similar results were reported in a trial of gantenerumab, another human anti-Aβ monoclonal antibody, where patients receiving the drug at 60 mg (N = 6), 200 mg (N = 6), or placebo (N = 4) underwent PiB-PET at baseline and posttreatment (up to 7 monthly infusions) (Ostrowitzki et al., 2012). Mean PiB SUVr posttreatment was on average +11.0% of baseline in the placebo group, +2.1% in the low-dose treatment group, and –9.4% in the high-dose treatment group. While small and laden with caveats, these studies illustrate proof-of-concept for a very important translational application of amyloid PET. Ultimately, lower fibrillar Aβ burden will need to be linked to improved cognitive and functional outcomes for amyloid PET to be adopted as a true surrogate outcome measure in AD drug development.

XVI. F-18 Compounds

PiB is the most widely studied amyloid imaging agent and the first Aβ selective radiotracer to differentiate AD patients from HCs by *in vivo* PET imaging (Klunk et al., 2004). However, the short radioactive half-life of carbon-11 (about 20 min) limits the use of PiB only to those PET imaging centers with onsite capability to synthesize this radiotracer. Fluorine-18 (F-18) labeled PET tracers are longer lived (about 110 min) so they can be distributed to distant PET imaging sites. Several new [F-18]-labeled amyloid ligands have been developed recently for *in vivo* imaging of Aβ pathology. These radioligands include [F-18]flutemetamol, [F-18]AV-45 (florbetapir), [F-18]AV-1 (florbetaben), [F-18]AZD4694, and [F-18]FDDNP, and currently several are under development for use as clinically approved Aβ-imaging radiopharmaceuticals.

A. [F-18]Flutemetamol

[F-18]Flutemetamol is a 3'-fluoro analog of PiB (3'-F-PiB) currently being examined in Phase III FDA clinical trials. Being structurally similar to PiB, [F-18]flutemetamol was expected to demonstrate comparable brain uptake and clearance. Indeed, initial PET imaging studies show that compared to PiB [F-18]flutemetamol has similar retention characteristics

although somewhat more pronounced retention in white matter. A phase I clinical study of eight mild AD patients (MMSE 20–26) and eight HCs reported that [F-18]flutemetamol regional standardized uptake value ratios (SUVRs) were significantly higher in the neocortex and striatum of AD patients, while the values measured in white matter, cerebellum, and pons were not different from HCs (Nelissen et al., 2009).). In a multicenter phase II trial of [F-18]flutemetamol, Vanderberghe and colleagues studied 27 early AD, 20 amnestic MCI, 15 controls >55 years of age, and 10 controls <55 years of age, and reported 93.1% sensitivity and 93.3% specificity for AD (Vanderberghe et al., 2010). The same study reported a strong correlation (0.89–0.92) between [F-18]flutemetamol and PiB regional SUVRs in 20 AD and 20 MCI subjects (Vanderberghe et al., 2010). These data indicate that [F-18]flutemetamol is comparable to [C-11]PiB in its ability to detect brain fibrillar Aβ pathology in living subjects. In further support of this, Wolk et al. (2011) provided histopathological evidence in seven subjects who had a frontal cortical biopsy (as part of a clinical work-up for suspected normal pressure hydrocephalus) and later underwent [F-18]flutemetamol PET imaging, similar to previous reports of brain biopsy using PiB (Leinonen et al., 2008). They reported that a higher [F-18]flutemetamol uptake in frontal cortex correlated with amyloid plaque load determined using Aβ immunohistochemistry or thioflavin S staining in the frontal biopsy samples, further supporting that [F-18]flutemetamol is sensitive in detecting fibrillar Aβ plaques *in vivo* (Wolk et al., 2011).

B. [F-18]Florbetapir

[F-18]Florbetapir {(E)-4-(2-(6-(2-(2-(2-[F-18]-fluoroethoxy)ethoxy)ethoxy)pyridin-3-yl)vinyl)-N-methyl benzenamine; [F-18]AV-45; or amyvid} has proven to be effective in imaging Aβ fibrillar pathology *in vivo* (reviewed by Lister-James et al., 2011). Preclinical studies characterized postmortem binding of [F-18]florbetapir to Aβ plaques and demonstrated prominent *in vitro* labeling in brain tissue sections from AD patients but not in sections from control brains (Choi et al., 2009; Lin et al., 2010). A clinical trial performed on 18 mild-moderate AD patients (mean MMSE = 19.3) and 16 HCs showed that cortical regions had a higher [F-18]florbetapir retention, while white matter and cerebellar retention were not different between AD and control subjects (Wong et al., 2010). An analysis of multicenter PET data from 210 participants, pooled from four registered phase I and II trials of [F-18]florbetapir imaging, reported that positive PET scans indicative of fibrillar Aβ pathology were observed in 85% of 68 subjects with clinically probable AD, 47% of the 60 MCI subjects, and 28% of the 82 cognitively HCs >55 years old (Fleisher et al., 2011). [F-18]florbetapir PET scans were negative in all young subjects aged <50 years (n = 74) and there was a good correlation between [F-18]florbetapir retention *in vivo* and postmortem

measures of Aβ immunoreactive and neuritic plaques in a cohort of 29 terminally ill patients with mixed diagnoses who were evaluated with [F-18] florbetapir PET and later came to autopsy (Clark et al., 2011). However, there was a substantial variability in ratings of PET scans by independent readers in that study. Neuropathological confirmation of increased [F-18] florbetapir uptake in areas of neocortex, striatum, and thalamus which contained heavy loads of fibrillar Aβ deposits was also reported in a [F-18]florbetapir imaged patient with Down syndrome and AD (Sabbagh et al., 2011). New preliminary data demonstrate high sensitivity (92%) and specificity (91%) using quantitative assessment of global cortical SUVr to differentiate AD subjects from HCs, and indicate that [F-18]florbetapir PET is suitable biomarker for routine clinical use (Camus et al., 2012).

C. [F-18]Florbetaben

[F-18]Florbetaben {(E)-4-(2-(4-(2-(2-(2-[F-18]fluoroethoxy)ethoxy)ethoxy)phenyl)-vinyl)-N-methyl-benzenamine; [F-18]AV-1 or BAY-94-9172} is another [F-18]-labeled radioligand that is one atom chemically different from [F-18]florbetapir and in early PET brain scan clinical studies proved to be able to discriminate a group of 15 AD patients with significantly higher neocortical retention from 15 HCs and 5 FTLD cases (Rowe et al., 2008). A large multicenter phase II study of [F-18]florbetaben was conducted in 81 clinical probable AD patients and 69 HC subjects, and it showed 80% sensitivity and 91% specificity for distinguishing the AD group from controls (Barthel et al., 2011a). An exploratory, open-label, nonrandomized, single-center phase 0 study of [F-18]florbetaben PET imaging in 10 clinically probable AD and 10 HCs reported 90% sensitivity and 90% specificity (Barthel et al., 2011b). A recent review of three clinical studies involving 109 subjects with clinical diagnoses of AD, MCI, and various non-AD dementias (FTLD, VaD, DLB, PD) who were imaged with [F-18]florbetaben revealed that AD patients had significantly higher gray matter retention values (SUVRs), indicating higher Aβ burden, compared to other disease groups (Villemagne et al., 2011b). Florbetaben findings in DLB, PD, and MCI were similar to those previously described for PiB.

D. [F-18]FDDNP

[F-18]FDDNP (2-(1-{6-[(2-[F-18]fluoroethyl)(methyl)amino]-2-naphthyl} ethylidene)malononitrile) is a lipophilic tracer which binds in histological and autoradiography assays not only to aggregated Aβ in plaques but also to NFT (Agdeppa et al., 2001). PET imaging studies demonstrated that regional increases in [F-18]FDDNP uptake correlate with greater brain atrophy (i.e., lower MRI volumes) and reduced brain glucose metabolism (lower FDG-PET) in brain areas containing both Aβ plaques and NFT (Shoghi-Jadid et al., 2002;

Small et al., 2006). Several subsequent *in vivo* imaging studies compared [F-18]FDDNP to PiB retention in cognitively impaired subjects and HCs. Using both radiotracers, Shin et al. imaged 10 clinical AD and 10 HCs, and demonstrated that [F-18]FDDNP and PiB retention patterns were similar in the neocortical regions; however, in the mesial temporal lobe structures, known to contain large amounts of neurofibrillary pathology in AD (Braak & Braak, 1991), [F-18]FDDNP binding was strongest while PiB retention was minimal (Shin et al., 2008). Tolboom and colleagues examined 14 clinical probable AD, 11 amnestic MCI, and 13 HCs with both PiB and [F-18]FDDNP PET scans performed on the same day for most of the subjects (Tolboom et al., 2009). Global cortical uptake values of PiB and [F-18]FDDNP correlated directly but there were different regional binding patterns and PiB was better in detecting differences among clinical groups; although with both tracers, AD and MCI groups had higher global cortical uptake when compared to control values, only PiB showed no overlap between AD and control groups. These observations suggested that PiB and [F-18]FDDNP detect different but related pathology in the brain (Tolboom et al., 2009), in agreement with the idea that [F-18]FDDNP is valuable in detecting NFT pathology in addition to aggregated Aβ (Shin et al., 2011).

Preclinical characterization of the novel fluorinated PET radioligand candidates AZD2184 and AZD4694 demonstrated their high specificity for Aβ plaques in brain tissue sections from AD cases and transgenic APP mice (Johnson et al., 2009; Juréus et al., 2010). Full reports of the properties of these two radiotracers in detecting and assessing Aβ plaque deposits in PET human imaging studies have not been published to date.

Further studies in large numbers of subjects representing different clinical categories are required to characterize the existing radiotracers and develop new radiotracers for imaging the distribution and quantity of AD lesions in living subjects. Single or multiple tracer imaging studies using [F-18]-labeled PET radioligands will be extremely important and will complement clinical neurocognitive testing, making possible earlier and more sensitive detection of AD pathology as well as for monitoring disease progression and effects of new drug treatments.

XVII. Detection of the Earliest Signs of Amyloid Deposition

Since the initial PiB-PET studies, the focus of many research studies has shifted away from the robust signal seen in symptomatic AD and toward detection of the earliest signs of fibrillar Aβ pathology in cognitively normal individuals (see above, Section VI). This shift toward initial detection has generated a need for reliable methods that can distinguish brains free of fibrillar Aβ from brains that have early-stage fibrillar Aβ deposition. It is important that such methods can be standardized and applied across many centers.

It should be noted that PiB retention is a continuous measure and need not necessarily be dichotomized into PiB(+) and PiB(–). Many studies have used PiB retention as a continuous variable, correlating PiB retention to a variety of cognitive or biochemical measures (Bourgeat et al., 2010; Forsberg et al., 2010; Furst et al., 2010; Mormino et al., 2009; Pike et al. 2007; Rentz et al., 2010; Resnick et al., 2010). This approach may be preferred for some applications; however, in other applications it is necessary to dichotomize subjects into PiB(+) and PiB(–). This may be most important in the cognitively normal subjects when attempting to disentangle the effects of normal aging from the effects of preclinical AD (Sperling et al., 2011).

A variety of *ad hoc* objective approaches have been presented to define an amyloid-positive cutoff using amyloid imaging. These methods include using one or two standard deviations above the mean of the control data (Edison et al., 2008; Kemppainen et al., 2007; Klunk et al., 2004 Okello et al., 2009); inspection of quantitative PET data for natural breakpoints in the distribution of tracer retention in combinations of young controls, elderly controls and/or AD patients (Edison et al., 2008; Gomperts et al., 2008; Hedden et al., 2009; Jack et al., 2008; Maetzler et al., 2009; Mintun et al., 2006; Mormino et al., 2011; Morris et al., 2010; Rowe et al., 2007; Roe et al., 2008); the low end of the range of tracer retention in clinically (Sperling et al.,2009) or pathologically (Fleisher et al., 2011) defined AD patients; receiver operating characteristic (ROC) analyses of PET data from control and AD subjects (Devanand et al., 2007; Mormino et al., 2009; Ng et al., 2007; Pike et al., 2007); visual reads (Engler et al., 2007; Gomperts et al., 2008; Johnson et al., 2007; Ng et al., 2007; Rabinovici et al., 2007; Suotunen et al., 2010; Tolboom et al., 2009); and cluster analysis methods using both PiB(+) and PiB(–) elderly control subjects (Bourgeat et al., 2010). Each approach has advantages and shortcomings. Most of these approaches involve subjective choices such as the number of standard deviations above the control mean, the exact location of the natural breakpoints and the interpretation of the visual read. Others, like ROC analysis or using the low end of the AD range, rely on the composition of the AD group, which can vary widely depending on the nature of the particular control or AD population utilized. Methods that rely on analysis of the entire control group can result in cutoffs that are unduly affected by the amyloid-positive high outliers in the control group (e.g., control mean + standard deviations). While many of these methods yield similar results, further study will be required to identify a widely applicable and standardized method to identify both the earliest signs of Aβ deposition and Aβ deposition that is clinically meaningful, or "AD-like."

XVIII. Limitations, Validity, and Unresolved Questions

While amyloid imaging represents a major advance in AD research, the field is still young and there are a number of unresolved questions

and limitations. The dynamic range, threshold and ceiling effects, binding interactions as well as the relative selectivity of amyloid tracers for different tertiary structures of Aβ deposits remain works in progress. Roughly 10–20% of clinically diagnosed AD patients are amyloid-negative (Fleisher et al., 2011; Rabinovici et al., 2011; Rowe et al., 2010; Vandenberghe et al., 2010; Villemagne et al., 2011b), and while some of these may have been clinically misdiagnosed, a case report of deficient *in vitro* PiB retention to an otherwise typical AD postmortem brain (Rosen et al., 2010) suggests that there are factors other than low Aβ burden that can lead to a negative *in vivo* study. Methodologically, the fundamental factors impacting white matter binding are incompletely understood (Fodero-Tavoletti et al., 2009). Furthermore, the relative benefit and optimal methodology for implementing partial volume correction are actively being debated. Partial volume effects are important to consider when quantifying binding in the atrophic brain, where low counts in enlarged CSF spaces can dilute signal from gray matter. This is a particularly relevant issue for quantifying amyloid in longitudinal studies, when progressive brain atrophy can be expected (Jack et al., 2009). Partial volume effects from white matter may be an issue for F-18 tracers, for which the dynamic range in white matter is similar to or even exceeds the dynamic range of gray matter (Baker et al., 2012). While most studies have employed cerebellar gray matter as the reference region for normalizing counts across subjects, some argue for inclusion of white matter (to account for the variability of white matter binding across subjects) (Clark et al., 2011) or even for a combined cerebellum-pons region that would be less susceptible to mis-registration errors when defined on a structural MRI (Koeppe, 2012).

In terms of translational applications, the relative advantages of qualitative visual versus quantitative classification are still being weighed. Visual interpretations may be easier to implement on a broad scale in the clinical arena. Quantitative methodologies are more objective, but also more prone to misclassification due to partial volume effects or errors in automated processing. The optimal threshold for defining a scan as positive (visually or quantitatively) is a moving target, as discussed in detail above, and will likely differ depending on whether the goal is early detection (more liberal threshold) or ruling-in AD as the cause of cognitive impairment (more conservative threshold). Whether and how the threshold should be adjusted for patient variables such as age, sex, education, and ApoE genotype is an open question. It is still not clear whether dichotomizing scans as positive or negative will be sufficient for clinical purposes, or whether there is additional information to be attained from the degree and spatial distribution of tracer binding. Only limited studies have directly compared the relative merit of amyloid PET to CSF biomarkers, MRI, FDG, or clinical measures in common clinical scenarios. Further, the

data that are available about the clinical utility of amyloid imaging are almost entirely derived from highly selected research cohorts, and it is not yet clear how the technique will perform in typical clinical populations. Finally, even in scenarios where amyloid imaging will very likely yield helpful diagnostic and prognostic information (e.g., MCI, atypical dementia in a young patient), it is not at all clear that third party payers will cover the cost of PET unless a clear benefit in clinical outcome can be demonstrated.

XIX. Conclusion

PiB-PET and Aβ imaging mark a major advancement in the study of the pathology and treatment of AD. One facet of Aβ deposition that has become clear from PiB-PET studies is how early in the spectrum of AD the full burden of amyloid plaques begins to develop. Therefore, a major challenge of amyloid imaging is and will be how to determine the earliest signs of amyloid accumulation, its association with cognitive impairments and, ultimately, whether or not this early amyloid deposition will invariably lead to clinical dementia in a high percentage of individuals. This will likely require the field to focus on cognitively normal elderly and detection of the earliest signs of amyloid deposition, in order to determine the clinical significance of presymptomatic pathology. As anti-amyloid therapies are developed, it will be critical to effectively identify the earliest changes in amyloid deposition and the clinical significance of such changes. Further, as has been reflected in the new diagnostic criteria for AD, MCI, and "preclinical AD," the use of amyloid imaging, alone or in conjunction with other biomarkers, will likely be critical to the identification of subjects at risk for AD and future decline.

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List of Abbreviations

[F-18]	fluorine-18
[F-18]FDDNP	2-(1-{6-[(2-[F-18] fluoroethyl) (methyl)amino]-2-naphthyl}ethylidene)malononitrile
[F-18]florbetaben	(E)-4-(2-(4-(2-(2-(2-[F-18]fluoroethoxy)ethoxy) ethoxy)phenyl)-vinyl)-N-methyl-benzenamine; [F-18]AV-1 or BAY-94-9172
[F-18]florbetapir	(E)-4-(2-(6-(2-(2-(2-[F-18]-fluoroethoxy)ethoxy) ethoxy)pyridin-3-yl)vinyl)-N-methyl benzenamine; [F-18]AV-45; or amyvid
[F-18]flutemetamol	2-{3-[18F]fluoro-4-(methylamino)phenyl}-6-hydroxybenzothiazole; or 3'-Fluoro-PiB
[F-18]THK523	2-(4-aminophenyl)-6-(2-fluoroethoxy)quinoline)
[H-3]	hydrogen-3 (Tritium)
6-CN-PiB	6-cyano-PiB
AD	Alzheimer's Disease
ApoE	apolipoprotein E
APP	Aβ precursor protein
Aβ	amyloid-β
BACE1	beta-secretase 1
CAA	cerebral amyloid angiopathy
CDR	clinical dementia rating
CERAD	Consortium to Establish a Registry of Alzheimer's Disease
CJD	Creutzfeldt–Jakob disease
CSF	cerebrospinal fluid
CVD	cardiovascular disease
DLB	dementia with Lewy bodies
DVR	distribution volume ratios
ELISA	enzyme-linked immunosorbent assay
eoFAD	early-onset familial Alzheimer's disease
FDG	fludeoxyglucose
FDG-PET	FDG-positron-emission tomography
FTD	frontotemporal dementia
FTLD	ftrontotemporal lobar degeneration
FUS	fused-in sarcoma
HC	healthy control
K_d	dissociation constant
K_i	inhibition constant
LB	Lewy bodies
lvPPA	logopenic-variant primary progressive aphasia

MCI	mild cognitive impairment
MMSE	mini-mental status exam
MRI	magnetic resonance imaging
NFT	neurofibrillary tangles
NIA-RI	National Institute on Aging and Reagan Institute
PCA	posterior cortical atrophy
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PET	positron-emission tomography
PiB	Pittsburgh compound-B ([N-methyl-^{11}C]2-(4'-methylaminophenyl)-6-hydroxybenzothiazole; or [^{11}C]6-OH-BTA-1)
PiB(–)	PiB-negative
PiB(+)	PiB-positive
PiB-PET	PiB-positron-emission tomography
PS1	presenilin-1
p-tau	phosphorylated tau
rCMRglc	regional cerebral metabolic rate of glucose
ROC	receiver operating characteristic
SUV	standardized uptake value
SUVr	standardized uptake value ratio
TDP-43	TAR DNA-binding protein 43
ThT	thioflavin-T
VaD	vascular dementia

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Mitochondrial Abnormalities in Alzheimer's Disease: Possible Targets for Therapeutic Intervention

Abstract

Mitochondria from persons with Alzheimer's disease (AD) differ from those of age-matched control subjects. Differences in mitochondrial morphology and function are well documented, and are not brain-limited. Some of these differences are present during all stages of AD, and are even seen in individuals who are without AD symptoms and signs but who have an increased risk of developing AD. This chapter considers the status of mitochondria in AD subjects, the potential basis for AD subject mitochondrial perturbations, and the implications of these perturbations. Data from multiple lines of investigation, including epidemiologic, biochemical, molecular, and cytoplasmic hybrid studies, are reviewed. The possibility that mitochondria could potentially constitute a reasonable AD therapeutic target is discussed, as are several potential mitochondrial medicine treatment strategies.

I. Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia. In the United States, it is estimated that one out of every eight persons over

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the age of 65 suffers from AD, and almost half of those over the age of 85 are affected (Evans et al., 1989; Thies & Bleiler, 2011). It has also been recognized for some time, as Alois Alzheimer's first reports were presented and published at the start of the twentieth century (Alzheimer, 1907, 1911; Alzheimer et al., 1995). Many academic clinicians and scientists focus on AD, and industry maintains active AD drug development and testing programs.

All this helps create the false impression that we truly understand what AD is, what causes it, and how to effectively treat it. On the contrary, how we even define the disease is somewhat arbitrary, and this really has been the case since the term "Alzheimer's disease" was first proposed.

By the late nineteenth century, it was recognized that with advancing age, the brain cortex of some animal species develop extracellular protein accumulations called plaques (Blocq & Marinesco, 1892). During the first decade of the twentieth century, this phenomenon was also noted to occur in the brains of elderly humans, and that this histological change was often associated with dementia, a clinical syndrome characterized by declining cognitive function (Fischer, 1907; Redlich, 1898). At this same time, Alois Alzheimer reported the brains of several relatively young, or "presenile," demented individuals also developed plaque deposits (Alzheimer, 1907, 1911). Alzheimer further described intracellular protein aggregations which he called tangles. Because dementia was relatively common in those reaching old age, affected persons were not felt to have an actual disease, even when plaques and tangles were present (Kraepelin, 1910). Such persons were simply felt to have a senile dementia syndrome that frequently accompanies old age. It was only those with presenile dementia, plaques, and tangles who actually qualified for an AD diagnosis.

Over the next 100 years, much was learned about the structural basis of the plaques and tangles. The major protein in the plaques is folded in an amyloid configuration (Divry, 1927), and is called beta amyloid (Aβ) (Glenner & Wong, 1984). Aβ arises as a degradation product of a larger protein called the amyloid precursor protein (APP) (Kang et al., 1987). The tangles contain aggregated assemblies of a protein called tau, and tau protein in tangles is heavily phosphorylated (Grundke-Iqbal et al., 1986).

During the second half of the twentieth century, the clinical definition underwent significant revision. The distinction between when a demented person with plaques and tangles was young enough to have AD or old enough to have age-associated senile dementia had always been somewhat arbitrary (Swerdlow, 2007a). To minimize the impact of this distinction (Katzman, 1976), the original AD subjects were stated to have presenile dementia of the Alzheimer's type, while the elderly subjects were said to have senile dementia of the Alzheimer's type. However, the age boundary between the presenile and senile conditions was still arbitrary, and most reverted to simply calling the clinical syndrome AD, regardless of age.

In the early 1990s, it was shown that mutations in the APP gene, which resides on chromosome 21, cause brain disease in general and can also cause an AD presentation characterized by progressive dementia, plaques, and tangles (Goate et al., 1991; Levy et al., 1990). This discovery gave rise to a hypothesis, the amyloid cascade hypothesis, that posited AD was itself induced by the presence of Aβ-containing amyloid plaques (Hardy & Allsop, 1991).

It was subsequently discovered that mutations in two other genes, the presenilin 1 (PS1) and presenilin 2 (PS2) genes, caused an AD presentation and that the presenilin proteins contributed to APP processing (Kimberly et al., 2000; Levy-Lahad et al., 1995; Sherrington et al., 1995; Wolfe et al., 1999). Aβ was found to be toxic under cell culture conditions (Yankner et al., 1989), and although belief that plaques drove AD neurodysfunction and neurodegeneration gradually fell out of favor, modified versions of the amyloid cascade hypothesis in which different preplaque Aβ configurations were deemed the critical toxic moiety increasingly came to dominate the field (Hardy & Selkoe, 2002; Walsh & Selkoe, 2007). Consistent with this view, transgenic mouse models that developed cortical plaques were created and became the mainstay of preclinical AD research (Hsiao et al., 1996).

Along the way, clinically based AD concepts began to clash with the amyloid cascade hypothesis. The most important discrepancy arose from the fact that plaques are often observed in the brains of the nondemented elderly, a finding not entirely consistent with the idea that Aβ is the primary disease mediator (Swerdlow, 2011a). Recently, this has been administratively addressed by expanding the definition of AD to include anyone with brain plaques, regardless of clinical status. Those with plaques and dementia are now said to have AD, while those with plaques and no clinical signs can be diagnosed with "preclinical AD" (Sperling et al., 2011).

So, despite the fact that many people are diagnosed with it, many investigators study it, and much has been written about it, what we now call AD remains a somewhat arbitrary construct whose definition is subject to change. In essence, the same controversies that were identified over 100 years ago remain. We still do not know whether AD is a single homogeneous entity or a collection of clinically and histologically overlapping conditions. The relationship between brain aging and AD is unclear. Whether Aβ truly induces a disease-driving cascade in all or even some patients remains unproven. To date, a number of therapeutic interventions that benefit AD transgenic mice have been shown not to benefit affected patients, which raises the question of how well these mice model human AD (Holmes et al., 2008; Swerdlow, 2007a). With this in mind, this chapter will now address the role of mitochondria in AD and the possibility that mitochondria might offer a potential AD therapeutic target.

II. Mitochondrial Function in AD

AD is usually thought of as a disease of the brain. Biochemical changes, though, are certainly not brain-limited (Swerdlow, 2012). Systemic mitochondrial changes between the mitochondria of AD and age-matched control subjects have been observed.

A. Brain

1. *Morphology*

Many of the changes typical of compromised mitochondria are seen in the AD brain. For example, disruption of mitochondrial cristae and intramitochondrial accumulations of osmiophilic material are prevalent in AD brains compared to controls (Baloyannis, 2006, 2011; Saraiva et al., 1985). There is an increased range of mitochondrial sizes, with more enlarged mitochondria but also elevated numbers of exceptionally small mitochondria (Hirai et al., 2001). Overall, the average size of AD neuron mitochondria is smaller than it is in control brain neurons (Baloyannis, 2006; Hirai et al., 2001).

2. *Mass*

How mitochondrial mass changes in AD brains is not straightforward. Using PCR-based approaches to quantify mitochondrial DNA (mtDNA) reveals that AD subject brain cortices contain lesser amounts of amplifiable mtDNA (Brown et al., 2001; de la Monte et al., 2000). The simplest explanation for this is that AD brains have reduced amounts of mtDNA, and by extension a reduced mitochondrial mass. However, in a study in which a labeled oligonucleotide probe was used to detect mtDNA in hippocampal neurons, a complex picture emerged (Hirai et al., 2001). When only mtDNA within normal-appearing mitochondria were considered, less mtDNA was revealed. A large number of mitochondria were also found within phagosomes, and these degrading mitochondria also hybridized the mtDNA oligonucleotide probe. When this additional mtDNA was taken into account, the AD hippocampal neurons actually contained more mtDNA.

Mitochondrial mass has been assessed using alternative approaches, including an immunochemical quantification of mitochondrial-localized proteins. In one study, an antibody probe to an mtDNA-encoded cytochrome oxidase (COX) subunit, COX1, was found to be increased in AD brain hippocampal neurons (Hirai et al., 2001). In a different study, tangle-free hippocampal neurons showed more COX1 and COX4 staining, while staining was markedly reduced in tangle-bearing neurons (Nagy et al., 1999). Other authors reported cytochrome COX protein in general was reduced in AD brain homogenates (Kish et al., 1999).

Although it could only be used as a very indirect index of mitochondrial mass, mtDNA expression in the form of mitochondrial RNA has also been evaluated. Northern blot-based studies found some, but not all, mitochondrial RNA transcripts were reduced (Chandrasekaran et al., 1994). Similarly, nuclear-encoded oxidative phosphorylation subunit expression is reduced (Liang et al., 2008). Findings from other studies, though, suggest a more complex picture. For example, Manczak et al. reported COX subunit expression was actually increased in at least some AD brain neurons (Manczak et al., 2004). The authors concluded this upregulation might represent a compensatory response to perturbed COX function.

Electron microscopy (EM) has been used to quantify AD neuron mitochondria. Several studies have reported the number of normal-appearing mitochondria was decreased (Baloyannis, 2006; Hirai et al., 2001). In one study, there was a concomitant increase in mitochondria located within phagosomes (Hirai et al., 2001).

The aggregate of these studies suggest that within the brain the number of normal-appearing mitochondria is diminished. Whether this reflects increased turnover, decreased synthesis, or both is not entirely clear. Potentially pertinent to this question, two relatively recent studies measured protein levels of peroxisome proliferator activated receptor gamma coactivator 1α (PGC1a), a transcriptional coactivator that serves as a master regulator of mitochondrial mass (Qin et al., 2009; Sheng et al., 2012). Both of these studies found that PGC1a levels were reduced in AD brains.

3. *Enzymes*

In the AD brain, particular enzymes that mediate glucose metabolism may have either altered amounts or altered Vmax activities. For example, the neuronal enolase is more highly expressed and also shows a high degree of oxidative modification (Butterfield & Lange, 2009).

Within the mitochondria themselves, the measured activities of pyruvate dehydrogenase complex (PDHC) and the Krebs cycle enzyme α-ketoglutarate dehydrogenase complex (KDHC) are reduced (Gibson, Sheu, & Blass, 1998). The KDHC activity reduction is likely a consequence of posttranslational modification due to oxidative stress (Shi et al., 2011). The activity of isocitrate dehydrogenase, another proximal Krebs cycle enzyme, is reduced. Activities of enzymes in the distal Krebs cycle, including succinate dehydrogenase and malate dehydrogenase, are increased (Gibson, Starkov, Blass, Ratan, & Beal, 2010).

Regarding the electron transport chain (ETC), COX activity has consistently been observed to be lower in AD subject brains than it is in control subject brains (Swerdlow, 2012; Swerdlow & Kish, 2002). Histochemical approaches reveal AD brain hippocampi contain higher numbers of COX-activity-deficient neurons (Cottrell et al., 2001). Some studies have suggested neuroanatomically limited reductions and are consistent with the

possibility that the COX activity deficit is due to reduced synaptic activity (Simonian & Hyman, 1993). Other studies have utilized spectrophotometric Vmax measurements from brain homogenates (Swerdlow & Kish, 2002). In one study, dividing the COX Vmax to the density of a COX protein subunit on a Western blot rendered the activity comparable to that of the corrected control group activity (Kish et al., 1999). The authors concluded that reduced COX in AD brains is a consequence of reduced COX enzyme. Another study, though, found that COX activity when divided by the amount of spectrally determined COX was still low (Parker & Parks, 1995). It was further demonstrated that COX kinetics were altered and that the holoenzyme's low Km binding site was absent. These data argue that COX is structurally altered in the AD brain.

4. mtDNA

As discussed under the mitochondrial mass section, the case has been alternatively made that the amount of mtDNA in AD neurons is decreased or increased. These findings are not as diametrically opposed as it may seem. The amount of mtDNA may vary from neuron to neuron, and may further associate with the health of the neuron. Healthier neurons may have an increased amount of mtDNA, while more affected neurons may have decreased amounts. Certainly, in AD brain hippocampi, the number of neurons that show succinate dehydrogenase activity but which lack COX activity is increased (Cottrell et al., 2001). Because succinate dehydrogenase is entirely encoded by nuclear genes while COX contains mtDNA-encoded subunits, this suggests AD neurons have abnormally high levels of perturbed or mutated mtDNA, or else a severe state of mtDNA depletion.

In addition to changes in the quantity of mtDNA, differences in the quality of mtDNA have been reported. A probe specific to mtDNA containing the 5kDa common deletion found AD brain hippocampal neurons contained markedly increased amounts of this deletion (Hirai et al., 2001). Other studies using different approaches have also found that relative to control brains, AD brains contain increased amounts of the 5kDa deletion (Corral-Debrinski et al., 1994; Hamblet & Castora, 1997).

Oxidative modifications of the mtDNA are increased in AD brains, as evidenced by higher levels of 8-hydroxy-2-deoxyguanosine (2DG) (Mecocci et al., 1994). Oxidation-related nucleotide modifications can induce replication errors and an accumulation of somatic mutations. Some AD brain studies that surveyed mtDNA protein coding genes have reported a quantitative increase in the number of these mutations, although others have not (Chang et al., 2000; Lin et al., 2002). Another study determined levels of low-abundance heteroplasmic mutations in the mtDNA D-loop control region. Specific mutations were found in AD brains that were not present in control brains, and the overall burden of control region mutations was markedly

increased in the AD brains (Coskun et al., 2004). This study also reported reductions in an mtDNA-derived transcript, as well as a decreased mtDNA-to-nuclear DNA ratio.

While some have focused on characterizing presumably somatic, acquired mutations, others have probed whether inherited mtDNA sequences and mutations are present. To date, particular homoplasmic mtDNA mutations have been reported in AD subjects, but the causality of these mutations has been virtually impossible to prove (Swerdlow, 2012). In any event, inherited homoplasmic mtDNA mutations are at most an extremely rare cause of AD.

mtDNA sequences between individuals are extremely variable to begin with (Lu et al., 2010), and series of linked sequence deviations that can vary between populations and members of populations have been used to define mtDNA haplogroups (Torroni et al., 1996). Haplogroup sequence changes present in blood are also present in the brains of those who carry them, and haplogroups are amenable to association studies. A number of mtDNA haplogroup studies have reported associations between particular mtDNA haplogroups and AD (Swerdlow, 2012). Some studies have found certain haplogroups associate with an increased risk of AD, while other studies have found that other haplogroups associate with a decreased risk of AD. Although findings from some populations have also been found in others, the results of haplogroup association studies in AD have generally been inconsistent. To summarize, the presence of at least a small effect of mtDNA haplogroups on AD risk remains a possibility.

5. *Fission/Fusion*

Impaired mitochondrial dynamics have been widely implicated in neurodegenerative disorders such as AD (Chan, 2006a; Su et al., 2010). Mitochondria can undergo consecutive cycles of fusion, in which physically separate mitochondria link to produce a single organelle. This is counterbalanced by the process of fission, which features the breakdown of a single mitochondrion into smaller mitochondria (Detmer & Chan, 2007). These phenomena rely on a large group of conserved proteins, the dynamin-related GTPases.

Mitochondrial fission is crucial for mitochondrial renewal, redistribution, and proliferation within synapses, whereas mitochondrial fusion facilitates mitochondrial movement and distribution across axons and to the synapses themselves (Chen et al., 2007; Hoppins et al., 2007; Santos et al., 2010). A balance between these two events is crucial to maintain mitochondrial functional integrity, especially in neurons where mitochondrial fission and fusion are mandatory for the formation of synapses and dendritic spines (Arduino et al., 2011). Fusion is orchestrated by the mitofusins Mfn1 and Mfn2, which are responsible for outer membrane fusion, and optic atrophy 1(Opa1), which participates in the fusion of outer and inner membranes.

Fission requires Drp1 in mitochondria, and membrane constriction is facilitated by Fis1 (Chan, 2006b; Yoon et al., 2003).

Analyses of AD brains show down regulation of the Mfn1, Mfn2, and Opa1 fusion genes and increased expression of the Fis1 fission gene (Manczak et al., 2011; Wang et al., 2009). The status of the other fission-mediating protein, Drp1, is less clear as levels have been reported to be both reduced and increased (Manczak et al., 2011; Wang et al., 2009). Regardless, Drp1 activity is inactivated by S-nitrosylation (SNO-Drp1), and Cho and colleagues reported high levels of SNO-Drp1 in brain biopsies from AD subjects (Cho et al., 2009). Perturbed Drp1 function could lead to the production of functionally impaired mitochondria, and ultimately reduce synapse energy supplies (Barsoum et al., 2006).

Drp1, Opa1, Mfn1, Mfn2, and Fis1 proteins redistribute so that they accumulate in the cell soma. Neuronal processes are therefore depleted of these fission–fusion proteins (Wang et al., 2009). The authors of this study also manipulated mitochondrial fission–fusion proteins in M17 cells and hippocampal primary neurons, and found this affected intracellular mitochondrial distributions. They concluded that altered mitochondrial fission–fusion protein dynamics may play an important role in mitochondrial distribution and, consequently, synaptic dysfunction in AD neurons.

When primary cortical neuron cultures are exposed to S-nitrosocysteine (SNOC), a nitric oxide (NO) donor, uncontrolled fission occurs and this appears to represent an upstream and early event in neuronal cell death (Barsoum et al., 2006). Further, when mitochondrial fission is blocked by expression of the dominant negative Drp1K38A, cell death is reduced. In a similar type of study, when cultured cerebrocortical neurons were exposed to Aβ, S-nitrosylation of Drp1 occurred and resulted in the formation of SNO-Drp1 dimers (Westermann, 2009). This pattern is similar to what is found in the brains of human AD patients (Cho et al., 2009).

6. *Transport*

The delivery of mitochondria to regions of the neuron with high bioenergetic demand is required for proper functioning of neuron (Hollenbeck & Saxton, 2005; Li, Okamoto et al., 2004; MacAskill et al., 2010; Mattson et al., 2008). This mitochondrial transport is impaired in a number of neurodegenerative disorders. For example, it has been reported that mutant huntingtin protein in Huntington's disease (HD) enhances mitochondrial Drp1 activity, disrupts mitochondrial trafficking, and induces mitochondrial fission (Reddy & Shirendeb, 2011; Shirendeb et al., 2012). Anterograde mitochondrial transport is significantly reduced in neuronal cultures of superoxide dismutase (SOD)1-mutant mice, suggesting that impaired mitochondrial trafficking is an early event in amyotrophic lateral sclerosis (ALS) (De Vos

et al., 2007). Additionally, mitochondrial transport is impaired in dopaminergic neurons from a Parkinson's disease (PD) mouse model (Sterky et al., 2011). Given the prevalence of altered mitochondrial transport in other neurodegenerative diseases, it is likely that this critical phenomenon is also impaired in AD.

Indeed, mitochondrial distribution is abnormal in AD brains (Wang et al., 2009). One study showed that mitochondrial transport in AD patient brains is significantly decreased compared to control brains (Dai et al., 2002). In an elegant study by Trimmer and Borland, fluorescently labeled mitochondria in differentiated cytoplasmic hybrid (cybrid) cell lines generated from AD patients displayed reduced trafficking to neurite-like processes compared to control cybrid lines (Trimmer & Borland, 2005). This study provides further evidence that mitochondrial transport may be impaired in AD. Additionally, this study suggests that impaired mitochondrial transport in AD is mediated by mitochondrial function itself and ultimately by mtDNA. Other studies suggest that mitochondrial transport is altered in cell cultures treated with Aβ (Calkins & Reddy, 2011; Wang et al., 2009) and in mouse models of AD (Calkins et al., 2011; Massaad et al., 2010; Pigino et al., 2003).

In general, it appears that Drp1 abnormalities may impair mitochondrial transport by perturbing a functional relationship that exists between Drp1 and the dynein–dynactin transport complex (Ishihara et al., 2009; Varadi et al., 2004; Wang et al., 2009). For example, in AD subject fibroblasts Drp1 expression is significantly lower than in control fibroblasts (Wang et al., 2008). This Drp1 down-regulation occurs in conjunction with an abnormal mitochondrial distribution, and restoring normal Drp1 levels to AD fibroblasts repairs their mitochondrial transport defect. Therefore, although mitochondrial transport is certainly difficult to study in the autopsy brain, data suggest that in AD-perturbed mitochondria, fission–fusion dynamics may contribute to the apparent presence of impaired mitochondrial transport.

7. *Oxidative Stress*

Reactive oxygen species (ROS) are a frequent by-product of electron leakage from the inner mitochondrial membrane during mitochondrial oxidative phosphorylation. It is estimated that up to 4% of O_2 used by mitochondria is converted to superoxide radical (Hansford et al., 1997; Inoue et al., 2003; Markesbery & Lovell, 1998; Morten et al., 2006; Turrens & Boveris, 1980), and that approximately 10^9 to 10^{11} ROS are produced per cell per day (Bonda et al., 2010; Feinendegen, 2002; Ji, 1999; Petersen et al., 2007). Under normal conditions, ROS are rapidly cleared to increasingly lesser reactive species by enzymes such as SOD1, SOD2, catalase, and glutathione peroxidase (GPx). When mitochondria are perturbed, however, ROS

production may exceed the cell's ability to neutralize them, resulting in oxidative damage to the cell (Smith et al., 2000). Aging itself is associated with elevated ROS production by mitochondria (Ames et al., 1995; Shigenaga et al., 1994), and accumulation of oxidative damage over time may contribute to the noted association between advancing age and AD.

Oxidative stress is thought to be an early manifestation of AD (Nunomura et al., 2001). Studies of postmortem AD brains indicate widespread oxidative damage. Four-hydroxynonenal and acrolein, which are aldehydes produced by lipid peroxidation, and isoprostanes, which are proinflammatory products of arachidonic acid peroxidation, are significantly elevated in hippocampi from AD brains (Markesbery & Lovell, 1998; Pratico et al., 1998; Sayre et al., 1997; Singh et al., 2010). This indicates that excessive lipid oxidation occurs in the AD brain. In AD brains, both nuclear and mtDNA and RNA also display evidence of oxidative damage (Gabbita et al., 1998; Mecocci et al., 1994; Nunomura et al., 1999). Brains from individuals affected with AD further display increased protein oxidation, as evidenced by carbonyl-alterations of specific proteins (Castegna et al., 2002; Castegna et al., 2002; Smith et al., 1991; Sultana et al., 2010).

Many studies suggest that oxidative damage is also present in individuals with mild cognitive impairment (MCI), a syndromic state that in many cases represents a very early AD clinical stage (Aluise et al., 2011; Butterfield, Reed, et al., 2006, 2007; Keller et al., 2005; Lovell & Markesbery, 2008; Markesbery & Lovell, 2007; Pratico et al., 2002). In fact, studies suggest that levels of oxidative markers directly correlate with severity of cognitive impairment as well as symptomatic progression from MCI to AD (Ansari & Scheff, 2010; Keller et al., 2005).

Extensive oxidative damage in AD brains likely has significant consequences for neurons, as oxidative modification of proteins and other molecular components can alter cell function (Butterfield et al., 1997; Lauderback et al., 2001; Subramaniam et al., 1997; Sultana & Butterfield, 2009). As a major source of ROS production, mitochondria themselves are at a risk of acquiring oxidative damage. As discussed earlier, the activities of certain mitochondrial enzymes including isocitrate dehydrogenase, PDHC, KDHC, and COX are significantly reduced in the AD brain (Aksenov et al., 1999; Bubber et al., 2005; Butterfield, Poon, et al., 2006; Gibson et al., 1998; Manczak et al., 2004; Yates et al., 1990). These enzyme impairments may represent a consequence or cause of ROS production, or both. For instance, COX dysfunction might further elevate ROS production by stalling electron transfer (Barrett et al., 2004; Skulachev, 1996; Sullivan & Brown, 2005; Sullivan et al., 2004). Thus, dysfunctional mitochondria in AD may give rise to and perpetuate a vicious cycle of oxidant production in which impairment of one mitochondrial enzyme elevates ROS production, which in turn impairs the function of other

mitochondrial enzymes, which in turn further increases ROS production (Bonda et al., 2010; Zhu et al., 2004).

8. Apoptosis

AD brains experience significant neuron loss, which likely contributes to an affected person's cognitive decline (Shimohama, 2000; Terry et al., 1991). While some neuron loss is due to necrosis, the rest is likely due to or else invokes aspects of apoptosis, a tightly regulated form of programmed cell death (Barinaga, 1998).

DNA fragmentation, as assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling staining, is a common hallmark of apoptosis. Neurons in AD brains display increased DNA fragmentation compared to control brains (Anderson et al., 1996; Broe et al., 2001; Colurso et al., 2003; Lassmann et al., 1995; Li et al., 1997; Smale et al., 1995; Su et al., 1994; Troncoso et al., 1996). Many of these studies also reveal morphologic changes associated with apoptosis including abnormal chromatin, an absence of nucleoli, and shrunken or irregular cell shapes (Shimohama, 2000). Other studies note an increased proportion of apoptotic to normal neurons (Broe et al., 2001). In AD cell death surveys, DNA fragmentation also associates with expression of c-Jun, which is typical of apoptotic neurons (Behl, 2000), and caspase proteins (Masliah et al., 1998). These studies suggest apoptosis pathways are activated in the AD brain.

Correspondingly, AD brains express significantly higher levels of the proapoptotic proteins Bak and Bad (Kitamura et al., 1998; Shimohama, 2000). Other studies suggest that AD brains display elevated proapoptotic Bax (Su et al., 1997). Caspases 3 and 6, which are apoptosis "executioner" caspases, are increased in AD brains (Avila, 2010; Guo et al., 2004; Masliah et al., 1998; Rohn, Head, Nesse, Cotman, & Cribbs, 2001; Selznick et al., 1999; Stadelmann et al., 1999), as are the initiator caspases 8 and 9 (Albrecht et al., 2007; Rohn & Head, 2009; Rohn et al., 2001; Rohn et al., 2002).

Further evidence that apoptotic events are more frequent in AD brains than in age-matched controls comes from experiments evaluating the presence of the cytoskeletal spectrin protein fodrin, which is cleaved early in the apoptotic cascade by caspases (Cribbs et al., 2004). Brains from AD patients display increased amounts of fodrin cleavage products (Ayala-Grosso et al., 2006; Masliah et al., 1991; Masliah et al., 1990).

Interestingly, evidence suggests that the proapoptotic shifts seen in AD subjects are not brain-limited. One study found that lymphocytes from AD patients were predisposed to apoptosis (Eckert et al., 2001). Another study reported increased fodrin cleavage in fibroblasts from AD patients (Peterson et al., 1991).

In summary, substantial data suggest that apoptosis is elevated in AD. This is not surprising given the other molecular and biochemical

perturbations observed in this disease. For example, oxidative stress can predispose cells to apoptosis (Buttke & Sandstrom, 1994; Ray, Huang, & Tsuji, 2012; Sandstrom et al., 1994). The prolific oxidative damage present in AD may, therefore, contribute to increased apoptosis.

B. Systemic

1. *Enzymes*

Reduced platelet COX activity is observed in subjects with AD and with MCI (Cardoso et al., 2004; Parker, et al., 1990; Valla et al., 2006). MCI is considered a transitional state between normal aging and AD (Morris et al., 2001; Padurariu et al., 2010). The platelet COX activity reduction is apparent in the setting of preserved COX subunit levels (Cardoso et al., 2004). One study has also reported that COX activity is reduced in AD subject fibroblasts (Curti et al., 1997).

KDHC catalyses a critical reaction in the Krebs cycle and is also important in glutamate metabolism (Blass et al., 1997). Its activity is reduced in AD brains. Cultured skin fibroblasts from sporadic AD subjects and AD subjects with PS1 mutations also show reduced KDHC activity (Blass et al., 1997; Sheu et al., 1994). In contradistinction to this, despite the fact that PDHC activity is reduced in AD brains, nonbrain tissues do not show reduced activity (Gibson et al., 1998).

ROS can modify the structure and function of cell proteins, lipids, and DNA (Facchinetti et al., 1998). ROS levels are controlled through the action of antioxidant enzymes, such as SOD1, SOD2, GPx, and catalase. It has been reported that MCI and AD subjects have lower plasma SOD and GPx activities than control subjects (Padurariu et al., 2010; Rinaldi et al., 2003). In erythrocytes from AD subjects, however, catalase and GPx activity were elevated. The activity of another antioxidant enzyme, glutathione reductase (GR), was reduced in both MCI and AD subjects (Torres et al., 2011).

2. *mtDNA*

Excess deletion mutations have not been demonstrated in AD subject's peripheral tissues. However, it is important to note that in general, deletions are uncommon in some peripheral tissues, such as blood, which are commonly studied because they are easy to collect. An increase in control region point mutations was reported in AD subject lymphocytes (Coskun et al., 2010). Interestingly, mtDNA haplogroup studies have utilized mtDNA from blood samples, and many of these studies have reported associations between haplogroups (Swerdlow, 2012).

Studies using cybrid cell lines consistently suggest that if mtDNA does in fact differ between AD and control subjects, then these differences are

not brain-limited (Swerdlow, 2012). Cybrid studies are performed by transferring mitochondria from a particular cell population to cell lines depleted of endogenous mtDNA. These mtDNA-depleted cell lines, referred to as ϱ0 cell lines, do not have a functional oxidative phosphorylation apparatus because they lack 13 crucial mtDNA-encoded proteins (7 from complex I, 1 from complex III, 3 from complex IV, and 2 from complex V). mtDNA contained within the transferred mitochondria populates the cell lines and restores their aerobic competence. The resulting unique cell lines are true cytoplasmic hybrids because they contain cytosolic components from two sources, the original ϱ0 cell line and the cells that provided their functional mitochondria (Fig. 1). Biochemical differences, although often subtle, are demonstrable between cybrid cell lines whose mtDNA is reconstituted from different sources. Because different cybrid cell lines prepared using the same parent ϱ0 line have identical nuclear DNA genes, and because cell lines are expanded and maintained under identical conditions, these biochemical differences presumably reflect and arise from differences in their mtDNA.

When COX activities between AD cybrid line series are compared to those of control cybrid cell line series, although considerable overlap

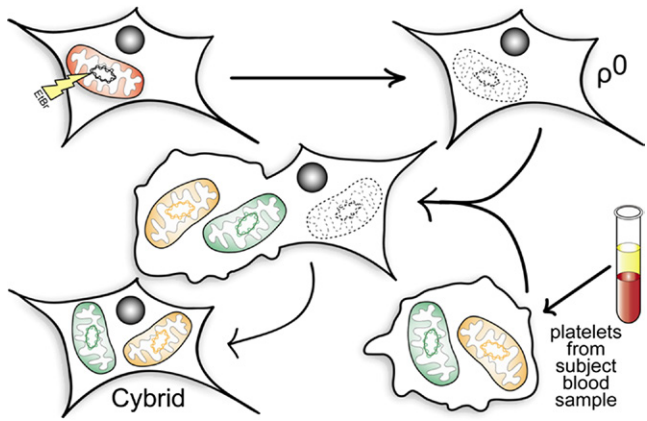

FIGURE 1 **Cytoplasmic hybrids.** ϱ0 cell lines are generated by chronically treating an established cell line with ethidium bromide (EtBr). This blocks mtDNA replication and leads to its total depletion from the EtBr-treated cells. Due to a lack of mtDNA-encoded oxidative phosphorylation complex subunits, the resultant cell lines are unable to complete electron transport chain transport and oxidative phosphorylation. The ϱ0 cells can then be fused with platelets isolated from patient blood samples to generate cytoplasmic hybrid (cybrid) cell lines. Cybrid lines contain mtDNA from the platelet donors, and nuclear DNA from the ϱ0 cell line. This relationship lets investigators study how specific mtDNA sequences affect cell bioenergetics, and how these effects influence downstream cell biochemical, molecular, and physiologic parameters. For color version of this figure, the reader is referred to the online version of this book.

between individual lines from each group are seen, the mean COX activity is characteristically lower in the AD cybrid cell lines (Swerdlow, 2007b, 2011b). This has been reported in multiple studies that have used ϱ0 cells prepared from SH-SY5Y neuroblastoma and NT2 teratocarcinoma cell lines. In these studies, the mtDNA used to restore mtDNA to the ϱ0 cells came from platelets obtained from human AD and control subjects. If these mean differences in fact are derived from mtDNA, it would indicate that mtDNA between age-matched individuals with and without AD does differ. It would further indicate that this mtDNA difference is not brain-limited, and extends at least to platelets and the megakaryocytes from which it derives.

Additional indirect support for systemic mtDNA differences between AD and non-AD individuals comes from AD endophenotype studies. An endophenotype is a partial or limited manifestation of a condition that is not sufficient to render a diagnosis of that condition. The presence of an endophenotype state does not necessarily indicate the affected individual will acquire the condition, although it does infer that the carrier individual has an increased risk of developing the condition.

In recent years, numerous studies have reported that AD-consistent endophenotypes can be demonstrated in the asymptomatic, middle-aged children of AD subjects. Interestingly, these endophenotypes are more profound in the children of AD mothers than they are in the children of AD fathers (Swerdlow, 2012). This suggests that although both parents contribute to AD risk, AD mothers contribute to a greater extent. This, in turn, implies that a maternally inherited genetic factor influences the development of AD (Mosconi, Bertia, et al., 2010).

These AD endophenotype studies have been conducted using metabolic, structural, and biochemical approaches. 2-deoxy-2 [F-18] fluoro-D-glucose positron emission tomography (FDG PET) studies show that the children of AD mothers, but not the children of AD fathers, have patterns of reduced glucose utilization that resembles patterns observed in AD subjects themselves (Mosconi et al., 2007, 2009). MRI studies show increased brain atrophy and rates of brain atrophy in the children of AD mothers as compared to the children of AD fathers (Berti et al., 2011; Honea et al., 2011; Honea et al., 2010). Oxidative stress and Aβ changes can be observed in the children of AD mothers (Mosconi, Glodzik, et al., 2010). As they age, the children of AD mothers accumulate greater amounts of Aβ in their brain parenchyma than do the children of AD fathers (Mosconi, Rinne, et al., 2010). In persons at risk for midlife cognitive softening due to the possession of an apolipoprotein E gene (APOE4) allele, those with an AD mother perform less well on memory tests (Debette et al., 2009). Finally, platelet COX activity is lower in the children of AD mothers than it is in the children of AD fathers (Mosconi et al., 2011). Collectively, these findings suggest a maternally inherited

TABLE I Effect of Maternal Influence on Nondemented Subject AD Endophenotypes

Endophenotype parameter	*Evaluated by*	*Change*
Brain glucose utilization	FDG PET	Reduced glucose utilization and more rapid glucose utilization decline rate in regions commonly affected in AD subjects
Brain volume	MRI with voxel-based morphometry	More atrophy and higher rates of atrophy in AD-affected regions
Brain Aβ	PET PIB	Increased brain parenchyma Aβ levels
CSF Aβ	CSF ELISA	Aβ42/Aβ40 ratio decreased
CSF isoprostanes	Mass spectrometry	Isoprostanes elevated
Memory performance	Cognitive evaluation	Among APOE4 carriers, lower memory test scores
COX activity	Platelet mitochondria COX Vmax assay	Reduced COX activity

genetic factor influences AD risk, and that this maternally inherited genetic factor is more likely to be mtDNA than an epigenetic or sex-linked factor (Table I).

3. *Fission/Fusion*

Fission/fusion dynamics are also altered in at least one nonbrain tissue of AD patients (Wang, Su, et al., 2008). Potential mechanisms that may underlie this phenomenon have been proposed and evaluated.

In one experiment, fibroblasts from non-AD subjects were treated with hydrogen peroxide (H_2O_2) in order to simulate a state of oxidative stress. This caused levels of the mitochondrial fission-promoting Drp1 protein to fall. Drp1 protein reduction, in turn, associated with a redistribution of mitochondria, and recapitulated changes that were observed in sporadic AD fibroblasts (Wang, Su, et al., 2008).

M17 cell lines that overexpress APP show mitochondrial fragmentation and redistribution of their mitochondria (Wang et al., 2008). Similarly, fibroblasts that overexpress wild type or mutant APP show a reduction in Drp1 and altered mitochondrial trafficking (Wang et al., 2008). In both sporadic AD fibroblasts and in M17 cells overexpressing APP, mitochondria cluster in the perinuclear region while the number of mitochondria in the cell periphery falls. Whether perturbed mitochondrial fission and fusion dynamics in sporadic AD subject fibroblasts is truly caused by Aβ overproduction is unclear, but the simple fact that mitochondrial fusion–fission dynamics are altered outside the brains of sporadic AD subjects contributes

to the increasing realization that at biochemical and molecular levels, AD is not a brain-limited disease.

4. *Oxidative Stress*

As previously discussed, brains from individuals with AD undergo extensive oxidative damage throughout the disease process. Significant evidence suggests that oxidative damage in AD is not brain-limited, but is also present systemically in AD patients (Burns et al., 2009).

One study evaluated the presence of oxidative stress in platelets and erythrocytes from normal controls and AD patients. This study found elevated oxidative stress markers in AD patients in the form of thiobarbituric acid-reactive substances, nitric oxide synthase activity, and Na,K-ATPase activity, suggesting that oxidative stress is present systemically in AD (Kawamoto et al., 2005). Another study found that ROS are elevated in circulating neutrophils from AD patients (Vitte et al., 2004). Plasma from AD subjects shows significantly decreased levels of the antioxidants lycopene, lutein, and carotene when compared to plasma from control subjects, and leukocytes from AD patients display elevated levels of oxidized DNA (Mecocci et al., 1998, 2002; Migliore et al., 2005; Morocz et al., 2002).

Oxidative stress is also ubiquitous in patients with MCI, suggesting that the oxidative damage seen in AD is a continuation of the stress that is also present during MCI. Interestingly, many studies further suggest that between MCI and AD subjects, no major differences in oxidative stress markers such as malondialdehyde and oxidized glutathione exist (Baldeiras et al., 2008; Bermejo et al., 2008; Padurariu et al., 2010). Rather, these studies propose that the primary biochemical differences between MCI and AD lie in the levels and activity of antioxidants such as SOD, glutathione peroxidase, and vitamin E. This suggests that a loss of one's ability to compensate for oxidative stress may underlie or else serve as a marker of MCI-to-AD progression.

Additional evidence suggests that oxidative stress markers may correlate with disease progression and severity in AD patients. Torres et al., 2011, recently found that plasma levels of malondialdehyde, a lipid peroxidation product, directly associate with impaired cognitive function in AD patients. The authors also found that the ratio of GR activity to GPx activity, which provides an indication of a cell's antioxidant capacity, associates with cognitive function (Torres et al., 2011). It has also been reported that in individual AD subjects, serum levels of vitamin E, a dietary antioxidant, relate to cognitive status (Baldeiras et al., 2008; Panza et al., 2010).

Data such as these have encouraged investigators to attempt to develop peripheral AD diagnostic and biomarker tests (Burns et al., 2009; Pratico, 2005). While a definitive biomarker with adequate sensitivity and specificity

remains to be identified, a plethora of data suggests that at least on a biochemical and molecular level, AD is a systemic disorder.

III. Mitochondria as a Therapeutic Target in AD

Accumulating data suggest mitochondrial function, if not changes in cell bioenergetics or the pathways that regulate cell bioenergetics, is perturbed early in the course of AD. In this respect, it is possible that at the commencement of AD itself mitochondria are altered by a more upstream process. If so, then treating mitochondrial abnormalities may benefit affected patients to some degree. It may also be the case that mitochondrial or bioenergetic dysfunction may actually constitute the primary, etiologic cause of AD (Swerdlow et al., 2010). If so, then therapies directed toward the mitochondria or cell bioenergetics could, should they target and remediate the primary problem, prove clinically transformative (Swerdlow, 2009, 2011c). To date, several clinical trials have evaluated agents that intended to target mitochondria or the consequences of mitochondrial dysfunction.

A. Overview of Mitochondrial Medicine

1. Historical Perspective

Mitochondrial medicine can be defined as any therapeutic intervention that specifically targets mitochondria themselves or specific consequences of mitochondrial dysfunction (Swerdlow, 2009). Mitochondrial medicine approaches were pioneered in rare diseases characterized by mitochondrial dysfunction and, in some cases, in rare diseases arising from identified mtDNA mutations (Luft, 1994).

For example, it has seemed obvious for some time that enhancing overall mitochondrial function might benefit patients with mtDNA diseases (Swerdlow, 2007c). Small studies have evaluated the effects of supplementing electron acceptor and donor molecules, such as coenzyme Q and menadione. The intent of such treatments has been to increase the passage of electrons through the ETC, or increase the COX-mediated delivery of electrons to molecular oxygen by bypassing upstream bottlenecks. Others have attempted to increase the flow of pyruvate-derived carbon into the Krebs cycle by activating PDHC. To accomplish this, investigators have administered drugs such as dichloroacetate that inhibit the PDHC kinase, which is an upstream inhibitor of PDHC. Other approaches have included raising the levels of PDHC cofactors, such as thiamine and lipoic acid (LA) (Swerdlow; Swerdlow, 2007c; 2009, 2011c).

Other classic mitochondrial medicine approaches intended to help maintain cell ATP levels have been tried in various degenerative mitochondriopathies. Creatine within cells binds high-energy phosphate groups,

initially generated by the mitochondrial ATP synthase (complex V), to form phosphocreatine. It has been postulated that increasing cell creatine levels will increase the levels of cell phosphocreatine, and that in the event that cell ATP levels are expended then the high-energy phosphocreatine phosphate group might be used to regenerate ATP. This has been tried in some neurodegenerative diseases including HD, PD, and ALS, but these trials have failed to show a meaningful benefit (Swerdlow, 2007c, 2009, 2011c).

Some mitochondrial medicine approaches have targeted the replacement of specific missing or depleted mitochondrial molecules, to some cases with great effect. Carnitine supplementation can prove transformative in cases of carnitine deficiency, just as coenzyme Q supplementation can greatly improve the clinical status of persons with coenzyme Q deficiency (Quinzii et al., 2007).

Although it is often not considered a "mitochondrial medicine" approach *per se*, interventions that target potential downstream effects of mitochondrial dysfunction have been tried in a variety of conditions. The most common of these targets has included the reduction of oxidative stress. While antioxidant clinical trials to date have not proved transformative in any trial, some trials have concluded particular antioxidant interventions may possibly confer, in some cases, a very limited therapeutic effect (Swerdlow, 2007c).

2. *Newer Strategies*

Recently, attempts have been made to make relatively nonspecific interventions more specific. For example, it has been posited that the failure of antioxidant therapies to truly benefit persons with mitochondrial disorders may relate to the fact that most antioxidants do not target free radicals within mitochondria themselves. This has justified the creation of mitochondrially targeted antioxidant molecules (Reddy, 2008; Reddy et al., 2012).

Other recently promoted strategies have sought to take advantage of drugs that have more general effects on mitochondrial physiology. Mitochondrial "stabilization" is one such effect. Under *in vitro* conditions, mitochondrial stabilization is typically defined as an induced perpetuation of the mitochondrial membrane potential under stress conditions, or as a preservation of mitochondrial size under stress conditions. Mitochondrial stabilization has been attempted in ALS. Development of the mitochondrial stabilizer minocycline, which interferes with mitochondrial permeability pore function, was terminated after trial results indicated accelerated decline in treated subjects (Gordon et al., 2007). Another mitochondrial stabilizer, R-pramipexole, suggested a therapeutic benefit and more definitive trials are now underway (Cudkowicz et al., 2011).

For cases in which mtDNA may initiate dysfunction, attempts have been made to manipulate mtDNA itself. Although protein nucleic acids (PNAs) were reported years ago to have the ability to strategically influence mtDNA replication under *in vitro* conditions (Taylor et al., 1997), this approach has had problems under more physiologic conditions. The feasibility of delivering mitochondrial-targeted restriction enzymes that degrade specific mtDNA sequences has also been shown in a number of studies (Wenz et al., 2010). One group has been working toward the development of mtDNA delivery systems that can deposit exogenous mtDNA payloads to mitochondrial matrices (Khan & Bennett, 2004).

Induction of mitochondrial biogenesis has been proposed for the enhancement of mitochondria function in conditions in which mitochondrial mass is reduced (Ghosh et al., 2007; Swerdlow, 2007c). Advocated strategies include increasing activity or levels of the transcription factor A of the mitochondria (TFAM) or PGC1a (Swerdlow, 2009, 2011c).

B. Track Record of Mitochondrial Medicine for AD

1. Oxidative Stress

Perturbed mitochondrial function can be associated with increased ROS production. Electrons that enter the ETC, when not added to molecular oxygen by COX to form water, can react with molecular oxygen in a less controlled fashion to produce the superoxide anion. The superoxide anion, in turn, can be converted to H_2O_2 and from there into other ROS species (Balaban et al., 2005; Fukui & Moraes, 2008). Some degree of physiologic ROS production occurs as a by-product of cell respiration, and in AD the rate of ROS production appears to be increased (Lin & Beal, 2006; Shi et al., 2008). Because of this, a number of investigators have proposed using approaches intended to decrease oxidative stress, such as the administration of antioxidant compounds (Aliev et al., 2009; Moreira et al., 2009).

Although several antioxidants have been tested in AD subjects, none have shown a robust effect (Swerdlow, 2011b). Discouragingly, successes obtained in studies of animal models tend not to be reflected in human trials. For example, administering vitamin E to Tg2576 mice before Aβ plaque deposition occurred suppressed brain lipid peroxidation and Aβ plaque deposition (Sung et al., 2004), which suggests vitamin E supplementation could potentially benefit AD pathology. Although a large human AD study that evaluated vitamin E at doses of 2000IU per day did report a possible slowing of clinical progression, this was only evident after the data were mathematically adjusted to account for the fact that at the start of the study, the treatment and placebo groups were not identical in terms of their cognitive abilities (Sano et al., 1997). Without this correction, no difference was observed. Although for some time after this trial high-dose vitamin E was

commonly offered to AD patients, a reassessment of vitamin E therapy concluded the adverse effects of this approach might outweigh its very limited (if any) benefits (Bjelakovic et al., 2007; Miller et al., 2005). Subsequently, it has become uncommon to prescribe high doses of vitamin E to AD patients.

LA, in addition to serving as a coenzyme for the mitochondrial pyruvate and α-ketoglutarate dehydrogenase complexes, has robust antioxidant properties (Moreira et al., 2009). Some clinical studies have reported AD subjects treated with LA showed a slowed rate of decline (Hager et al., 2007; Hager et al., 2001), but this finding has yet to be replicated in a large-scale trial.

Idebenone, a water-soluble synthetic analogue of CoQ10, has been evaluated in AD subjects (Chaturvedi & Beal, 2008; Senin et al., 1992). A 6-month placebo-controlled trial reported that idebenone-treated subjects showed less decline on the Alzheimer's Disease Assessment Scale (ADAS), a test of cognitive performance (Weyer et al., 1997). In another study performed by the Alzheimer's Disease Cooperative Study (ADCS) group, idebenone appeared to slow decline on the ADAS, but did not meaningfully benefit global function (Thal et al., 2003). The ADCS group idebenone trial was therefore interpreted as a negative trial.

The failure of antioxidants to clearly benefit AD patients may be due to several factors. One possibility is that in the human clinical trials, treatment was initiated too late in the course of the disease (Conte et al., 2004; Kamat et al., 2008; Sung et al., 2004). Other possibilities are that the compounds tested to date may have had limited brain penetration, or may have failed to reach mitochondria, the likely source of increased ROS production in AD (Manczak et al., 2010). To circumvent this, mitochondria-targeted antioxidants have now been developed. The most studied mitochondria-targeted antioxidant is MitoQ, which is generated through the covalent binding of ubiquinone, an antioxidant, to the triphenylphosphonium cation (TPP^+). This compound rapidly crosses the blood–brain barrier (BBB) and accesses neuron mitochondria (Bolognesi et al., 2009; McManus et al., 2011). In animal models, MitoQ has been shown to effectively reduce Aβ-induced oxidative stress and Aβ toxicity in neuron cultures, where it promotes neurite outgrowth and synaptic connectivity (Manczak et al., 2010). It has been shown to prevent cognitive decline in 3xTg-AD mice (McManus et al., 2011). MitoQ has been evaluated in a phase II trial of another neurodegenerative disease, PD, but the results of that trial were not encouraging.

Of course, another potential explanation for the failure of antioxidants thus far to demonstrate efficacy in AD is that oxidative stress may not be a major driver of neurodysfunction and degeneration in AD. If oxidative stress is a downstream consequence of mitochondrial dysfunction, remov-

ing it might do little to repair the underlying, more critical mitochondrial lesion.

2. *Electron Transport*

Small studies have evaluated the effects of thiamine and LA in AD subjects. In some cases, these cofactors have been used as part of a "cocktail" with other cofactors and vitamins (Blass & Gibson, 2006). Encouraging preliminary results have been reported using this strategy, but results from more conclusive studies are yet to appear. Some agents that may serve as ETC-associated electron donors and acceptors have undergone early-stage testing in humans, such as a methylene blue derivative (Wischik et al., 2008). In developing methylene blue for the treatment of AD, the responsible investigators have not identified the mitochondria as a potential target, but nevertheless under *in vitro* conditions methylene blue does appear to affect ETC electron transport (Atamna & Kumar, 2010; Atamna et al., 2008; Callaway et al., 2004).

Another approach for enhancing mitochondrial oxidative phosphorylation includes using ketone bodies such as beta-hydroxybutyrate (Swerdlow et al., 1989). The rationale underlying this approach is based on the observations that glucose utilization is reduced in the AD brain, and that ketone bodies constitute an alternative carbon source that can be used to support oxidative phosphorylation (Swerdlow, 2011b). More recently, a medium chain triglyceride (MCT) supplement has been marketed for the treatment of AD. As is the case with other MCTs, it is converted by the liver to beta-hydroxybutyrate and this elevates plasma beta-hydoxybutyrate levels. This strategy has been tested in humans with AD. While not conclusive, reported data do not exclude the possibility that some subjects benefit from this treatment (Henderson et al., 2009).

3. *Membrane Stabilization*

Latrepirdine (dimebon) is an antihistamine that was subsequently shown, under *in vitro* conditions, to have mitochondrial membrane stabilization properties (Bachurin et al., 2003; Zhang et al., 2010). Dimebon was studied in AD subjects. Although a phase II trial reported promising results (Doody et al., 2008), follow-up phase III studies did not replicate that finding and dimebon development efforts were terminated. Very recently, another mitochondrial membrane stabilizing agent, R-pramipexole, entered into an AD clinical trial.

C. Anticipated Mitochondrial Medicine Strategies

Some of the more recent mitochondrial medicine approaches listed above have been advocated and, in some cases, even attempted. Other very

unique mitochondrial medicine approaches have also been proposed and are being evaluated under preclinical conditions (Fig. 2).

1. Mitochondrial Mass Manipulation

Increasing mitochondrial mass for the treatment of AD was first proposed in 2007 (Ghosh et al., 2007; Swerdlow, 2007c). A rudimentary attempt to increase mitochondrial mass was previously attempted using the thiazolidinedione drugs rosiglitazone and pioglitazone. Although these drugs were originally considered for AD treatment based largely on their demonstrated anti-inflammatory effects, these drugs, which are used to treat type II diabetes, were subsequently shown to activate mitochondrial biogenesis signaling under preclinical testing paradigms. However, it is doubtful that they can reach levels within the brain that are high enough to activate mitochondrial biogenesis. Although the thiazolidinedione clinical trial data to date have not been uniformly negative, the overall impression these data give is that pioglitazone and rosiglitazone will provide no measurable benefit or, at best, an extremely small benefit

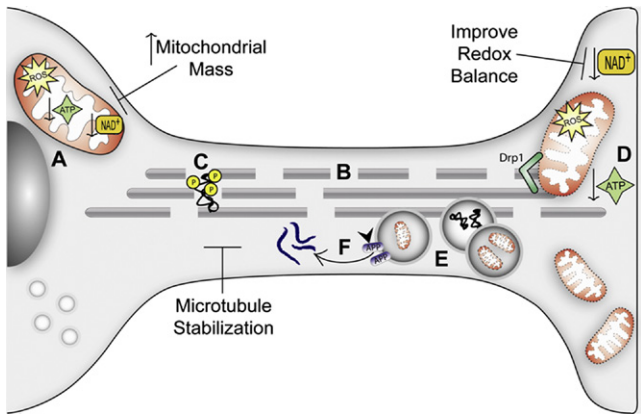

FIGURE 2 **Current and anticipated mitochondrial medicine strategies.** Dysfunctional mitochondria in AD result in reduced ATP availability (A), which promotes microtubule network breakdown (B), jeopardizing the transport of molecules and organelles along the cell. Further, it promotes tau dissociation from microtubules and its consequent hyperphosphorylation (C). These events compromise synapse energy supplies (D), and the transport of AVs toward the cell body (where lysosomes are located) is impeded (E). This promotes accumulation of Aβ aggregates, which may be formed by APP cleavage at AV membranes (F). Microtubule network stabilization may improve ALP function, promote transport along axons, and reduce Aβ production. Increasing mitochondrial mass may compensate for declines in mitochondrial function and their overall functional capacity. Shifting the cell redox balance to a more oxidized state may cause the cell bioenergetic infrastructure to function more efficiently, promote mitochondrial biogenesis, and activate pathways that allow cells to tolerate stress conditions. For color version of this figure, the reader is referred to the online version of this book.

(Geldmacher et al., 2011; Gold et al., 2010; Risner et al., 2006). In the meantime, other ways to manipulate mitochondrial mass are in various stages of development.

2. *Redox State Manipulation*

In this section, redox state refers to a cell's electron balance as defined by ratios of electron donor and acceptor molecule pairs. In this respect, an important indicator of a cell's bioenergetic state is the ratio defined by amounts of nicotinamide adenine dinucleotide's oxidized (NAD+) and reduced (NADH) derivatives.

While bioenergetics help determine a cell's redox state, a cell's redox state can also influence its bioenergetic function. Data demonstrating this latter point comes from multiple lines of investigation. Caloric restriction shifts the liver's redox balance toward a more oxidized state, and this is associated with mitochondrial biogenesis at least in the liver (Civitarese et al., 2007; Lambert et al., 2004; Lopez-Lluch et al., 2006) and also perhaps in the brain (Nisoli et al., 2005). Physical exercise, which should shift the muscle redox balance toward a more oxidized state, induces muscle mitochondrial biogenesis (Baar et al., 2002; Holloszy & Coyle, 1984; Hood, 2009).

Theoretically, enzymes that depend on NAD+ levels, such as silent mating type information regulation 2 homolog 1(SIRT1), should activate in the setting of increased NAD+ (Guarente, 2007; Haigis & Guarente, 2006). SIRT1 activation has been advocated for the treatment of several diseases, including AD (Anekonda & Reddy, 2006; Guarente, 2007; Haigis & Guarente, 2006). Polyphenol compounds are believed to work as sirtuin activators (Baur, 2010; Lagouge et al., 2006). An AD clinical that will evaluate the effects of resveratrol, a polyphenol, on AD clinical status is scheduled to be performed.

3. *Cytoskeletal Manipulation*

Mitochondrial dysfunction can induce cytoskeletal perturbations (Cardoso et al., 2010; Moreira et al., 2006). In the AD brain, reduced ATP levels may deregulate cytoskeleton homeostasis and microtubule integrity. Due to several unique neuron morphologic features, such as extended axons, branched dendritic arbors, and synaptic connections, both communication and continuity between a neuron's cell body and its distal regions must be adequately maintained. In this regard it is well known that neurons are highly sensitive to disturbances in microtubule-dependent transport (Trimmer & Borland, 2005).

AD neurons show cytoskeletal changes. Compared to brains from control subjects, microtubule assemblies are reduced (Cash et al., 2003; Santa-Maria et al., 2005). Surveys of AD brain pyramidal neurons suggest that changes in microtubule homeostasis precede neurofibrillary tangle (NFT)

formation (Cash et al., 2003). NFTs consist of the microtubule-associated protein (MAP) tau, which plays a role in microtubule function, neurite growth, and cytoskeleton maintenance (Stamer et al., 2002). Although a number of tau residues are phosphorylated under physiological conditions, in AD tau phosphorylation increases from 3- to 4-fold. Other studies show that tau overexpression alters cell shape, leads to a loss of polarization, and slows down cell growth. This is accompanied by a change in mitochondrial distribution; this change is characterized by organelle clustering (Ebneth et al., 1998).

The associated microtubule perturbations are accompanied by changes in the autophagic lysosomal pathway (ALP), or autophagy. Autophagy is a tightly regulated process that plays an important role in cellular maintenance. It ensures adequate levels of essential cell intermediates are maintained (Cuervo, 2004). The process begins with the regulated formation of a cytosolic membrane that encapsulates a region of the cytoplasm and its organelles within a double membrane called an autophagic vacuole (AV) or autophagosome (Levine & Kroemer, 2008). APP, which is a transmembrane protein, can be processed by the endosomal–lysosomal pathway which initiates when material internalized by endocytosis or pinocytosis is sorted into endosomes (Nixon, 2007). Data suggest that a change in the rate of autophagy, or the factors which cause AVs to accumulate, may contribute to Aβ overproduction in AD (Levine & Kroemer, 2008; Yu et al., 2004). Indeed, evidence from AD subject brains shows massive AV accumulation within dystrophic neurites occurs (Nixon et al., 2005; Yu et al., 2005).

Autophagy may therefore be simultaneously impaired and induced in AD. One potential explanation for this is that mitochondrial dysfunction may disrupt the microtubule cytoskeleton, and lead to impaired AV retrograde transport toward the cell body where lysosomes are located. In support of this, it has been shown that microtubule depolymerizing agents disrupt vesicular transport and induce rapid AV accumulation (Kochl et al., 2006). Vinblastine, which inhibits microtubule assembly, leads to microtubule depolymerization and prevents AV-lysosome fusion (Boland et al., 2008; Xie et al., 2010).

Recently, Miyasaka and colleagues (Miyasaka et al., 2010) reported that tau hyperphosphorylation is more likely a consequence, as opposed to a cause, of microtubule disruption. This is consistent with the view that when the microtubule network is disrupted, tau dissociates from microtubules and becomes accessible to the kinases that promote its hyperphosphorylation (Silva et al., 2011). For this reason, it was postulated that microtubule stabilizing agents could potentially reduce neuronal dystrophy (Lee et al., 1994; Silva et al., 2011).

Data consistent with this view come from experiments using taxol (paclitaxel), a microtubule polymerizing agent that has been shown to

mitigate AD-associated pathology in AD model systems. In rats, taxol was found to protect cortical neurons from $A\beta_{25-35}$ toxicity, decrease calpain activation, and decrease cdk5/p25 complex formation (Li et al., 2003). In other models, taxol pretreatment prevented tau hyperphosphorylation and reduced Aβ-induced apoptosis (Michaelis et al., 2002). In a hippocampal slice model of lysosomal dysfunction, it was found that pretreatment with TX67, an analogue of taxol, restored pre- and postsynaptic protein levels and reduced synapse damage (Butler et al., 2007).

Recently, Silva and coworkers demonstrated that in SH-SY5Y cells exposed to $A\beta_{1-42}$, the resultant mitochondrial dysfunction perturbs AV transport via a microtubule-dependent mechanism (Silva et al., 2011). Taxol prevents $A\beta_{1-42}$-induced disorganization of the tubulin cytoskeleton, which secondarily reduces both cytosolic and mitochondrial Aβ content by enhancing ALP function.

Taxol, though, does not robustly access the central nervous system (CNS) (Liu et al., 2002). A drug with taxol-like properties, NAP (davunetide), an eight amino acid peptide derived from the activity-dependent neuroprotective protein (ADNP), was recently shown to cross the BBB after systemic or intranasal administration (Gozes et al., 2005). Although NAP was first described as an antioxidant, it is now recognized that following cell internalization NAP interacts with the microtubule cytoskeleton (Divinski et al., 2004; Gozes & Divinski, 2007). NAP has been shown to reduce tau hyperphosphorylation and Aβ accumulation in both *in vitro* and *in vivo* AD models, and also benefit cognitive test performance in some of these models (Gozes & Divinski, 2004; Matsuoka et al., 2007, , 2008; Shiryaev et al., 2009; Vulih-Shultzman et al., 2007). Although its biological effects remain to be fully investigated (Shiryaev et al., 2011), based on encouraging preclinical data and an apparent lack of toxicity, NAP is now being tested in persons with AD and other disorders of the CNS (Greggio et al., 2011; Idan-Feldman et al., 2011; Javitt et al., 2011). A recent phase IIa clinical study reported that intranasal NAP improved memory performance in patients with an amnestic MCI syndrome, a frequent AD precursor state (Gozes et al., 2009).

IV. Conclusion

Many key questions about AD remain unresolved. There is no uniform agreement over whether AD is a homogeneous or a heterogeneous entity, how it relates to brain aging, or even what causes most of the cases. It is clear from a population perspective that mitochondria and mitochondria-related phenomena differ between those who do and do not have this disease. The importance of these mitochondrial and bioenergetic differences to AD, however it is defined, has been variably consid-

ered to be irrelevant or epiphenomenal, a mediator of disease pathology, a major mediator of disease pathology, or the actual initiating cause of the disease. This latter view is based on observations that distinct mitochondrial parameters are observed in subjects at all stages of AD, in persons at increased risk for developing AD, and that these differences are not brain-limited.

Unless mitochondrial changes turn out to be so far downstream of another critical disease-driving process that they are inconsequential to the condition, mitochondria and cell bioenergetics should constitute reasonable therapeutic targets. Some rudimentary attempts at mitochondrial medicine have been attempted in AD, with clinical results to date showing either no or perhaps minor beneficial effects. In the meantime, a more sophisticated and integrated view of how mitochondrial function, maintenance, and biogenesis play out in cells and relate to other aspects of cell function is emerging. Advances along this line will help to guide and refine the development of future mitochondrial medicine approaches. In coming years or perhaps decades, it will be interesting to see how mitochondria and cell bioenergetics-targeted interventions affect persons with AD.

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Abbreviations

2DG	8-hydroxy-2-deoxyguanosine
Aβ	beta amyloid
AD	Alzheimer's disease
ADAS	Alzheimer's disease assessment scale
ADCS	Alzheimer's disease cooperative study
ADNP	activity-dependent neuroprotective protein
ALP	autophagic lysosomal pathway
ALS	amyotrophic lateral sclerosis
APOE	apolipoprotein E gene
APP	amyloid precursor protein
AV	autophagic vacuole
BBB	blood–brain barrier
CSF	cerebrospinal fluid

CNS	central nervous system
CoQ	coenzyme Q
COX	cytochrome oxidase
Cybrid	cytoplasmic hybrid
Drp	dynamin-related protein
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EtBr	ethidium bromide
ETC	electron transport chain
FDG PET	2-deoxy-2 [F-18] fluoro-D-glucose positron emission tomography
Fis1	fission 1
GPx	glutathione peroxidase
GR	glutathione reductase
H_2O_2	hydrogen peroxide
HD	Huntinton's disease
KDHC	α-ketoglutarate dehydrogenase complex
LA	lipoic acid
MAP	microtubule-associated protein
MCI	mild cognitive impairment
MCT	medium chain triglyceride
Mfn	mitofusin
mtDNA	mitochondrial DNA
NAD	nicotinamide adenine dinucleotide
NFT	neurofibrillary tangle
NO	nitric oxide
Opa1	optic atrophy 1
PD	Parkinson's disease
PDHC	pyruvate dehydrogenase complex
PIB	Pittsburgh Compound B
PGC1a	peroxisome proliferator-activated receptor gamma coactivator 1α
PNA	protein nucleic acid
PS	presenilin
ROS	reactive oxygen species
SIRT1	silent mating type information regulation 2 homolog 1
SNO	S-nitrosylation
SNOC	S-nitrosocysteine
SOD	Superoxide dismutase
TFAM	transcription factor A of the mitochondria
TPP+	triphenylphosphonium cation
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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γ-Secretase as a Target for Alzheimer's Disease

Abstract

γ-Secretase is a protease complex responsible for cutting the transmembrane domain of the amyloid β-protein precursor (APP) to form the amyloid β-protein (Aβ), an aggregation-prone product that accumulates in the brain in Alzheimer's disease. As evidence suggests that Aβ is critical to Alzheimer pathogenesis, γ-secretase is considered a key target for the development of disease-modifying therapeutics. The protease complex cuts many other substrates, and some of these proteolytic events are part of signaling pathways or other important cellular functions. Among these, proteolysis of the Notch receptor is essential for signaling that is involved in a number of cell-fate determinations. Many inhibitors of γ-secretase have been identified, but it is clear that drug candidates for Alzheimer's disease should have minimal effects on the Notch signaling pathway, as serious safety issues have arisen with nonselective inhibitors. Two types of promising candidates that target this protease complex have emerged: the so-called "Notch-sparing" γ-secretase inhibitors, which block cleavage of APP selectively over that of Notch, and γ-secretase modulators, which shift the proportion of Aβ peptides produced in favor of shorter, less aggregation-prone species. The current status and prospects for these two general types of candidates will be discussed.

I. Introduction

γ-Secretase is a highly conserved membrane-embedded protease complex that carries out essential functions in cell and developmental biology. Initial interest in γ-secretase was inspired by its key role in the pathogenesis of Alzheimer's disease. However, parallel studies ultimately revealed that its

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proteolytic activity is also required in a signaling pathway, mediated through the Notch receptor, which regulates critical cell differentiation events in all multicellular organisms, in embryogenesis and adulthood. Thus, while γ-secretase has been a major target for the development of disease-modifying Alzheimer therapeutics, these agents should avoid affecting the essential signaling roles of the protease complex. This chapter describes the discovery of the γ-secretase complex, its roles in biology and disease, its biochemical properties, the development of inhibitors and modulators of the complex, and the potential of the enzyme as a therapeutic target for Alzheimer's disease.

II. γ-Secretase in Alzheimer's Disease

A key step in the pathogenesis of Alzheimer's disease is proteolysis of the amyloid β-protein precursor (APP) that results in the formation of the amyloid-β protein (Aβ), the principle protein component of the characteristic cerebral plaques of the disease (Goedert & Spillantini, 2006). Aβ is produced from APP first by the action of β-secretase, a membrane-tethered enzyme that resembles pepsin and other water-soluble aspartyl proteases (Cole & Vassar, 2008). This proteolysis leads to membrane shedding of the large luminal/extracellular APP domain. The 99-residue membrane-bound remnant is then cleaved in the middle of its transmembrane region by γ-secretase, releasing Aβ, as well as near the inner leaflet at the ε site, releasing the APP intracellular domain (AICD) (Fig. 1) (Weidemann et al., 2002). Rare mutations in the APP gene, found in and around the Aβ region, cause familial early-onset Alzheimer's disease, and these mutations alter the production of Aβ (increasing the proportion of 42-residue form, Aβ42, over the

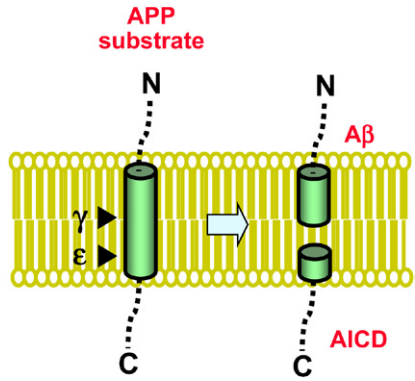


FIGURE 1 **The pathogenesis of Alzheimer's disease involves production of Aβ peptides.** Cleavage of APP substrate at the ε position releases the intracellular domain (AICD), while cleavage at the γ position releases aggregation-prone Aβ peptides. For color version of this figure, the reader is referred to the online version of this book.

40-residue form, Aβ40) or its aggregation properties, important evidence for the amyloid hypothesis of Alzheimer pathogenesis (Tanzi & Bertram, 2005).

The AICD proteolytic product has also been suggested to play a role in Alzheimer pathogenesis (e.g., Ghosal et al., 2009; Pardossi-Piquard et al., 2005). For instance, its release has been reported to enhance transcription of neprilysin (Belyaev et al., 2009; Pardossi-Piquard et al., 2005), an Aβ-degrading metalloprotease, although this has been called into question by another study (Hebert et al., 2006). In this model, decreased AICD production results in reduced levels of neprilysin and Aβ degradation, which may ultimately cause Alzheimer's disease. In contrast, an AICD transgenic mouse model suggests that increased levels of this APP metabolite, rather than decreases, can lead to Alzheimer-like characteristics (Ghosal et al., 2009). Whether AICD is reduced or elevated in Alzheimer's and is involved in any meaningful way in pathogenesis is unclear. However, it should be kept in mind that γ-secretase inhibition will reduce both Aβ and AICD, and reducing the latter may have unintended consequences.

Several contemporaneous observations provided critical clues for the identification of the elusive γ-secretase. First, genes encoding the nine-transmembrane-domain proteins presenilin-1 and presenilin-2 (PSEN1; PSEN2) were discovered in a search to identify other genes associated with familial, early-onset Alzheimer's disease. The disease-causing missense mutations were soon found to alter how γ-secretase cuts APP, leading to increased proportions of longer, more aggregation-prone forms of Aβ (Hardy, 1997). Second, knockout of *PSEN-1* dramatically reduced γ-secretase cleavage of APP (De Strooper et al., 1998). Third, the types of compounds that could inhibit γ-secretase contained moieties typically found in aspartyl protease inhibitors (Wolfe et al., 1999) (see Section V on inhibitors). These findings led to the identification of two conserved transmembrane aspartates in the multipass PSEN that are critical for γ-secretase cleavage of APP, suggesting that PSENs might be the responsible aspartyl proteases (Wolfe et al., 1999).

PSEN is cut into two pieces, an N-terminal fragment (NTF) and a C-terminal fragment (CTF), the formation of which is gated by limiting cellular factor(s) (Thinakaran et al., 1997). NTF and CTF remain physically associated in a high-molecular weight complex and are metabolically stable (Ratovitski et al., 1997; Yu et al., 1998). These and other results suggested that the NTF–CTF heterodimer is the biologically active form (Laudon et al., 2004). The NTF and CTF each contribute one of the essential and conserved aspartates, suggesting that the γ-secretase active site might be at the interface between these two PSEN fragments. In strong support of this hypothesis, transition-state analogue inhibitors of γ-secretase, designed to interact with the active site of the protease, bind directly to PSEN NTF and CTF (Esler et al., 2000; Li, Xu et al., 2000) (see Section V on inhibitors).

However, PSEN alone is not proteolytically active when overexpressed in cells. This fact, along with the requirement for other factors for PSEN NTF/CTF formation and the assembly of PSEN into large complexes, suggested that γ-secretase is composed of other subunits besides PSEN (see Section IV on the biochemistry of the γ-secretase complex).

III. γ-Secretase in Biology

At the same time *PSEN*s were discovered as susceptibility loci for Alzheimer's disease, they were also shown to be required for Notch signaling (De Strooper et al., 1999), a pathway essential for cell differentiation during development as well as in adulthood (Selkoe & Kopan, 2003). After Notch is synthesized in the ER, the receptor is cleaved in its extracellular domain during its passage through the secretory pathway, and the two pieces so generated remain associated. Upon interaction with a cognate ligand, Notch becomes susceptible to a second extracellular proteolysis, by a membrane-tethered metalloprotease, near the membrane. The membrane-associated remnant is then cleaved within its transmembrane domain by γ-secretase (De Strooper et al., 1999), releasing the Notch intracellular domain (NICD) (Fig. 2). NICD translocates to the nucleus and activates transcription after associating with the nuclear partner CSL (CBP/RBPjk, Su(H), Lag-1) (Schroeter et al., 1998). Knock-in of a Notch-1 transmembrane mutation greatly reduces PSEN-mediated proteolysis and leads to a lethal phenotype in mice similar to that seen in Notch-1 knockout mice, indicating that efficient γ-secretase cleavage is essential for Notch signaling during development (Huppert et al., 2000).

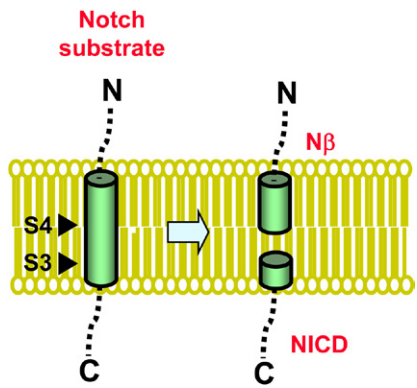


FIGURE 2 The Notch substrate is cleaved in at least two positions, at the S3 position to release the Notch intracellular domain (NICD) and at the S4 position to release a small peptide (Nβ). For color version of this figure, the reader is referred to the online version of this book.

Since the discovery that APP and Notch are cleaved by γ-secretase, a growing list of other substrates have been identified, including ErbB4, E- and N-cadherins, CD44, the low-density lipoprotein receptor, Nectin-1, and the Notch ligands Delta and Jagged (Haapasalo & Kovacs, 2011; Kopan & Ilagan, 2004). Knowledge of the cellular functions of these proteolytic events varies, but in the case of N-cadherin, the produced intracellular domain associates with the transcriptional activator CBP (CREB binding protein) and promotes its migration to the cytosol and degradation by the proteasome (Marambaud et al., 2003). Also, neuregulin-1-triggered cleavage of ErbB4 inhibits astrocyte differentiation by interacting with repressors of astrocyte gene expression (Sardi et al., 2006). While cellular function can be ascribed in some cases (Fig. 3, left), the ability of γ-secretase to cleave so many different substrates and its apparently poor sequence specificity suggests that a major role of this enzyme is to serve as a general degrading protease for membrane-bound protein remnants (Fig. 3, right) (Kopan & Ilagan, 2004). Indeed, γ-secretase appears to be unique among intramembrane proteases in its ability to process so many different substrates. The broad substrate recognition by γ-secretase is not well understood, but unlike the other intramembrane proteases, the enzyme apparently does not require helix-breaking residues near the cleavage sites within the substrates (Wolfe & Kopan, 2004). Thus, γ-secretase apparently has the ability to interact with hydrophobic helical transmembrane domains in general.

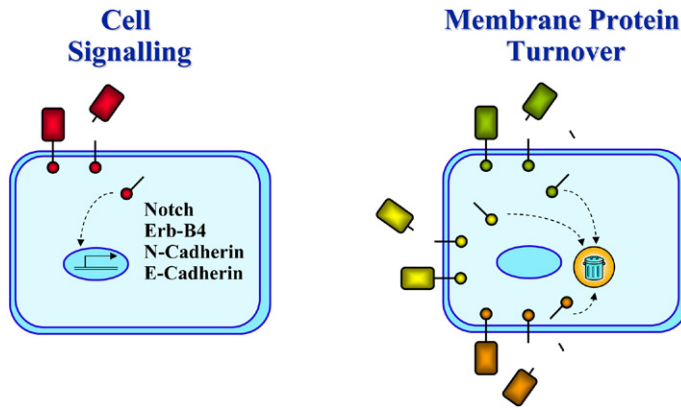


FIGURE 3 Left: Proteolysis by γ-secretase is involved in certain cell signaling events. After ectodomain shedding, typically by a membrane-tethered metalloprotease, substrate is cleaved by γ-secretase to release an intracellular domain that triggers transcriptional regulation. *Right:* Proteolysis by γ-secretase is involved in membrane protein turnover. Removal of protein stubs from the membrane by γ-secretase is followed by further degradation (e.g., by the proteasome, represented by the trash can). For color version of this figure, the reader is referred to the online version of this book.

IV. Biochemistry of the γ-Secretase Complex

The highly conserved role of γ-secretase in Notch signaling and its importance in the development made it possible to perform genetic screens in *Caenorabditis elegans* that identified two Notch modifiers, a single-pass membrane protein APH-2 (nicastrin), and a multipass protein APH-1 (reviewed in De Strooper, 2003; Spasic & Annaert, 2008). Nicastrin was independently isolated biochemically as a PSEN-associated protein and found to be essential for γ-secretase processing of both APP and Notch (Yu et al., 2000). A saturation screen in *Caenorabditis elegans* for *PSEN* modifiers identified these two proteins as well as Pen-2 (Francis et al., 2002). All four proteins (PSEN, nicastrin, Aph-1, and Pen-2) associate with one another and with an immobilized γ-secretase inhibitor (GSI) (Kimberly et al., 2003; Takasugi et al., 2003). Moreover, their coexpression increased γ-secretase activity in both *Drosophila* and mammalian cells and reconstituted activity in yeast (Edbauer et al., 2003; Hayashi et al., 2004; Kimberly et al., 2003; Takasugi et al., 2003; Zhang et al., 2005). Because yeast have no such protease activity and contain no apparent orthologs of these metazoan proteins, these findings strongly suggest that this quartet of proteins is necessary and sufficient for γ-secretase activity. This was subsequently confirmed through purification of the protease complex (Fraering et al., 2004).

Coexpression, RNA interference, and the identification of assembly intermediates suggest the order in which these four subunits come together, and partial dissociation, coimmunoprecipitations, and chemical crosslinking of the protease complex offer a model for how these subunits interact (Fig. 4) (reviewed in Spasic & Annaert, 2008; Wolfe, 2006, except for a more recent chemical crosslinking study by Steiner, Winkler, & Haass, 2008). Nicastrin and Aph-1 together can stabilize full-length PSEN, and final addition of Pen-2 triggers PSEN endoproteolysis and γ-secretase activity. Pen-2 is also required to stabilize the PSEN subunits. The specific biochemical functions of these PSEN cofactors have been mostly enigmatic; however, nicastrin has been suggested to play a role in substrate recognition (see later in this section).

Because it presumably contains water and uses hydrophilic residues, the membrane-embedded active site of γ-secretase should be sequestered from the hydrophobic environment of the surrounding lipid tails. Thus, the active site within PSEN was envisioned from the beginning to be part of a pore or channel that could allow entry of water (Wolfe et al., 1999). However, the substrate passes through the membrane and cannot enter such a pore or channel directly; docking on the outer surface of the protease, with lateral gating to bring the substrate into the internal active site, was thought to be required (Wolfe et al., 1999) (Fig. 4). Initial evidence for such a mechanism came from isolation of a γ-secretase substrate along with the

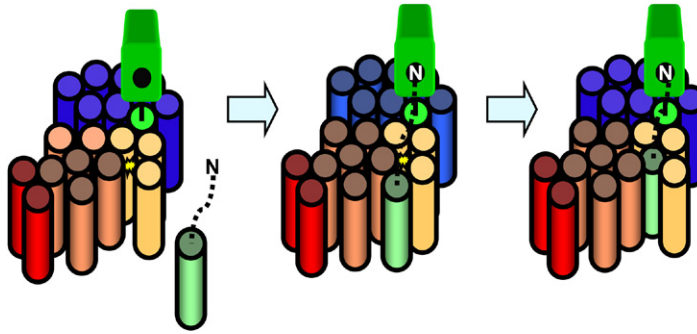


FIGURE 4 The γ-secretase complex is comprised of the integral membrane proteins Presenilin (as NTF and CTF subunits), Nicastrin, Aph-1, and Pen-2, with the active site inside Presenilin at the NTF–CTF interface. Membrane protein stubs serving as substrates dock both on the outer surface of Presenilin at the NTF–CTF interface and with the nicastrin ectodomain before entering into the internal active site. For color version of this figure, the reader is referred to the online version of this book.

protease complex using an immobilized transition-state analogue inhibitor (Esler, Kimberly et al., 2002). Substrate apparently bound to this special type of exosite, called the docking site, could copurify without being subject to proteolysis, because the active site is occupied by the resin-bound inhibitor.

Designed peptides based on the transmembrane domain of APP and constrained in a helical conformation can potently inhibit γ-secretase, apparently by interacting with this docking site (Das et al., 2003) (see Section V on inhibitors). Conversion of these helical peptide inhibitors into affinity labeling reagents led to the localization of the substrate docking site to the PSEN NTF–CTF interface (Kornilova et al., 2005). Transition-state analogue inhibitors also bind directly to the NTF–CTF interface, but at a site distinct from that of helical peptide inhibitors. These findings suggest a pathway for γ-secretase substrate from docking site to active site: upon binding to the outer surface of PSEN at the NTF–CTF interface, the substrate can pass, either in whole or in part, between these two PSEN subunits to access the internal active site (Fig. 4). Interestingly, extension of a ten-residue helical peptide inhibitor by just three additional residues resulted in a potent inhibitor (Bihel et al., 2004) apparently capable of binding both docking site and active site (Kornilova et al., 2005), suggesting that these two substrate binding sites are relatively close.

All interactions with the substrate seemed to be taking place on PSEN; however, one study has suggested that nicastrin also plays a critical role in substrate recognition (Shah et al., 2005). The ectodomain of nicastrin bears sequence resemblance to aminopeptidases, although certain catalytic residues are not conserved. Nevertheless, nicastrin may recognize the N-terminus of γ-secretase substrates derived from APP and Notch (Fig. 4),

and consistent with this notion, mutation of the aminopeptidase domain was reported to prevent this interaction. One conserved glutamate was noted to be especially important. The sequence of the substrate N-terminus is apparently not critical for the interaction, but a free amino group is. Indeed, simple formylation of the substrate N-terminus was enough to prevent effective substrate interaction and proteolytic processing. Thus, nicastrin may be a kind of gatekeeper for the γ-secretase complex: type I membrane proteins that have not shed their ectodomains cannot interact properly with nicastrin and do not gain access to the active site. However, a more recent study contradicts this view, with evidence that mutation of the conserved glutamate can interfere with the maturation of the γ-secretase complex, not with the activity of the mature complex (Chavez-Gutierrez et al., 2008).

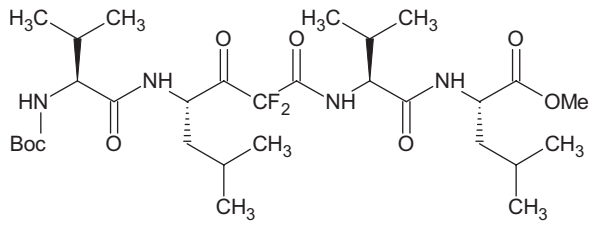
V. Inhibitors

The first reported γ-secretase inhibitors (GSIs) were peptide aldehyde-type calpain and proteasome inhibitors (Higaki et al., 1995; Klafki et al., 1996; Klafki et al., 1995). Despite their weak potency and lack of selectivity, these compounds were nevertheless the first chemical tools employed to address questions about γ-secretase. Because γ-secretase had yet to be isolated and identified, these compounds were tested in APP-transfected cells and found to increase levels of APP CTFs produced by α- and β-secretase (C83 and C99, respectively) and to inhibit the production of their γ-secretase cleavage products (p3 and Aβ, respectively). These compounds also revealed a pharmacological distinction between Aβ40 and Aβ42 production by γ-secretase (Citron et al., 1996; Klafki et al., 1996), a phenomenon since observed with many GSIs. Although this suggested distinct γ-secretases responsible for generating Aβ40 and Aβ42, subsequent work has demonstrated that this is not the case, as purification of tagged and overexpressed γ-secretase complexes of defined composition provides enzymes capable of generating both Aβ species (Fraering et al., 2004).

As peptide aldehydes typically inhibit serine and cysteine proteases, the fact that these compounds inhibited γ-secretase activity was initially interpreted as evidence that γ-secretases are in one or both of these protease classes. However, peptide aldehydes are readily hydrated to a form that resembles the transition state of aspartyl protease catalysis. Similarly, the first reported substrate-based inhibitor of γ-secretase activity, the difluoro ketone peptidomimetic compound **1** (also called MW167 and DFK167, Fig. 5) (Wolfe et al., 1998) could in principle inhibit a serine or cysteine protease in its keto form or an aspartyl protease in its hydrated form. However, difluoroalcohol analogues of **1** also could inhibit γ-secretase activity (Wolfe et al., 1999). As this class of peptidomimetic only inhibits

aspartyl proteases, by virtue of mimicking the transition-state of aspartyl protease catalysis, γ-secretase was suggested to be such a protease. Conversion of one of these difluoroalcohol peptidomimetics into an affinity labeling reagent led to the identification of PSEN1 NTF and CTF as the direct targets of this type of inhibitor (Esler et al., 2000). As difluoroalcohol peptidomimetics are transition-state analogues, this finding suggested that the active site of γ-secretase resides at the interface between these two presenilin subunits. Generation of a variety of such difluoroalcohols, varying in the identity of amino acid side chains, suggested that γ-secretase has relatively loose sequence specificity (Esler et al., 2004; Wolfe et al., 1999), a conclusion supported by later findings that the protease cleaves a wide variety of membrane proteins with no clear consensus sequence.

Another class of transition-state analogue inhibitor of aspartyl proteases, hydroxyethylamines, was found to inhibit γ-secretase activity in cell culture as well (Shearman et al., 2000). The most potent compound **2** (or L-685,458) (Fig. 5) was used to validate the isolation of γ-secretase activity in the detergent-solubilized state and demonstrated that immunoprecipitation of presenilin brought down γ-secretase activity (Li, Lai et al., 2000). As with the difluoroalcohols, conversion of this type of compound into affinity labeling reagents led to the identification of PSEN1 NTF and



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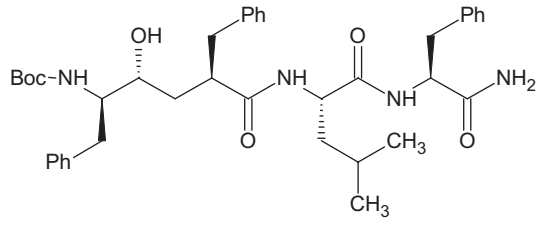


2

FIGURE 5 Transition-state analogue inhibitors of γ-secretase.

CTF as the direct target of this active site-directed inhibitor, and the potency and specificity of these affinity reagents allowed determination that full-length (i.e., unprocessed) PSEN1 was not a target, consistent with the holoprotein being a zymogen (Li, Xu et al., 2000). Use of a biotinylated version of **2** led to the isolation of γ-secretase with copurification of nicastrin and provided evidence for separate substrate binding and active sites (Beher et al., 2003).

Structurally related to the hydroxyethylamines are hydroxyethyl ureas, which replace one of the chiral backbone carbon atoms of the hydroxyethylamines with an achiral nitrogen. This subtle difference greatly simplifies the synthesis of these transition-state analogues, allowing facile generation of a variety of analogues for analysis of structure–activity relationships (Bakshi & Wolfe, 2004; Esler et al., 2004). In this way, the pockets in the protease active site that interact with the inhibitor side chains can be readily probed. Moreover, covalent attachment of one such compound to resin provided an affinity chromatographic method for isolating (Esler, Kimberly et al., 2002; Kimberly et al., 2003) and ultimately purifying γ-secretase (Fraering et al., 2004) demonstrating that the five components, PSEN1 NTF and CTF, Nicastrin, Aph-1, and Pen-2, are sufficient for protease activity and cleavage of APP and Notch substrates. The copurification of an endogenous APP substrate (C83) from the affinity matrix provided evidence for a substrate docking site on γ-secretase that is distinct from the active site, where the immobilized transition-state analogue binds (Esler, Kimberly et al., 2002), findings similar to those seen with an immobilized hydroxyethylamine inhibitor (Beher et al., 2003).

Another type of substrate-based GSI is the helical peptide. Because γ-secretase cleaves APP within its transmembrane domain and single transmembrane domains typically fold into a helical conformation, short peptides of 6–10 amino acids were synthesized that contained the γ-secretase cleavage sites of APP but with selected residues replaced with the helix-inducing aminoisobutyric acid (Aib) (Das et al., 2003). Surprisingly, D-peptides as well as L-peptides could potently inhibit γ-secretase activity, but in either case structural modifications that disrupt the helical conformation resulted in dramatically reduced potency. Extension of the D-peptide series led to the identification of a 13-residue helical peptide with an IC_{50} of 140 pM (Bihel et al., 2004).

This class of inhibitor was converted to affinity labeling reagents and, like the transition-state analogues, was found to directly bind to the PSEN1 NTF–CTF interface (Kornilova et al., 2005). However, competition experiments demonstrated that the helical peptide and transition-state analogue inhibitors bind to separate sites, consistent with the previous evidence for an initial substrate docking site (Beher et al., 2003; Esler, Kimberly et al., 2002). The finding that these two types of inhibitors bind to distinct sites at

the NTF–CTF interface suggests the substrate passes between the two PSEN1 subunits when transitioning from docking site to active site. Moreover, in contrast to a 10-residue helical peptide inhibitor, a 13-residue helical peptide inhibitor could compete for binding to PSEN1 with a transition-state analogue as well with its shorter 10-residue counterpart, suggesting that the active site and docking site are in close proximity (i.e., the length of the three extra residues). Helical β-peptides (containing β-amino acids) can likewise inhibit γ-secretase and compete with Aib-containing helical α-peptide affinity probes for binding to PSEN1; that is, these β-peptides also apparently bind to the initial substrate docking site (Imamura et al., 2009).

Another early prototype peptide-based inhibitor is compound **3** (or DAPT) (Fig. 6). This dipeptide analogue was the result of medicinal chemistry optimization of an initial hit from a high-throughput screening campaign (Dovey et al., 2001) and became an important research tool in the study of γ-secretase. Compound **3** showed good inhibitory potency (IC_{50} for Aβ lowering in cell-based assays of 20 nM) and was the first compound reported to be orally active *in vivo*, capable of lowering brain Aβ levels in an APP transgenic mouse model with an ED_{50} of 100mg/kg. The conversion of this compound into a photoaffinity labeling reagent led to the identification of the PSEN1 CTF as the direct target (Morohashi et al., 2006). This labeling could be blocked by transition-state analogue **2** or a helical peptide but only at elevated concentrations, suggesting that the binding site for **3** is distinct from the active site or the docking site, although it may overlap somewhat with these other sites. In this scenario, **3** may bind in the "transit path" between initial substrate docking site and

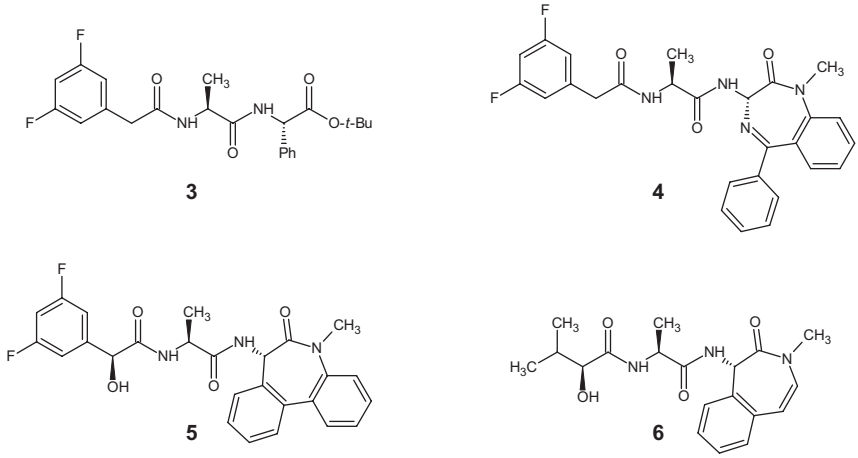


FIGURE 6 DAPT and related analogues.

active site. Related to **3** is the highly potent **4** (or compound E) (Fig. 6), in which the phenylglycine moiety is replaced by a benzodiazepine (Seiffert et al., 2000). This compound could inhibit Aβ production in cells with an IC_{50} of 300 pM. Surprisingly, a photoaffinity probe based on **4** labeled PSEN1 NTF but not the CTF, suggesting different binding pockets for the C-terminal phenylglycine of **3** and the C-terminal benzodiazepine of **4** (Fuwa et al., 2007). Nevertheless, because of their structural similarity and their ability to effectively block labeling by each other's photoaffinity probes, the binding sites for these two compounds are likely to be otherwise closely similar.

Further modification of **4** led to the exquisitely potent and *in vivo* active compound **5** (or LY-411,575) (Fig. 6). With an IC_{50} for inhibition of cellular Aβ production of 30 pM and good drug-like properties, **5** was highly effective in reducing brain Aβ levels in APP transgenic mice upon oral dosing ($ED_{50} < 1$mg/kg) (May et al., 2002). However, this compound also illustrated the toxicity issues that might be expected of a GSI with no selectivity for APP proteolysis vis-à-vis Notch. After treatment with **5** over the course of 15 days, gastrointestinal bleeding and immunosuppression due to peripheral inhibition of Notch signaling was observed (Wong et al., 2004). Despite this ominous result, nonselective GSIs of this type continued to be pursued on evidence from animal studies that careful dosing could identify a therapeutic window (e.g., Hyde et al., 2006).

Further modifications of **5** resulted in **6** (LY-450139, semagacestat) (Fig. 6), a compound that advanced into phase III clinical trials, even though phase I and II trials demonstrated lowering of steady-state Aβ levels in the plasma but not in the cerebrospinal fluid (Fleisher et al., 2008; Siemers et al., 2005, 2007). The phase III trial revealed severe gastrointestinal toxicity, immunomodulation, and skin cancer, effects expected from inhibition of Notch proteolysis and signaling (reviewed in Imbimbo et al., 2011). Also of concern was the finding that cognition in the drug-treated group worsened compared to placebo-treated, raising the possibility that lowering brain Aβ levels [or elevating APP CTF substrate (Mitani et al., 2012)] may be the cause. However, as **6** is a nonselective GSI, the negative effect on cognitive function is more likely attributable to blocking the proteolysis of another substrate besides APP, stressing the need to identify selective inhibitors toward the development of AD therapeutics.

Two other nonselective inhibitors are of particular note, as they were employed as chemical probes for investigation of γ-secretase biology. One is the benzodiazepine **7** (or compound D) (Fig. 7) developed by what was then Dupont Pharmaceuticals (Seiffert et al., 2000) (since acquired by Bristol-Myers Squibb). Radiolabeling of this compound provided a tool to visualize the binding sites in rodent brain, which were found in the olfactory bulb, cerebral cortex, hippocampus, and cerebellum (Yan et al., 2004). Brain regions labeled by **7** correlated with regions of PSEN1 gene expression. The

other useful probe is the caprolactam succinimide **8** (or compound C) (Fig. 7) developed by Dupont Pharmaceuticals. This GSI was among the first to be converted into an affinity probe and shown to directly bind to presenilin (Seiffert et al., 2000).

Some inhibitors have been reported to display selectivity for PSEN1-containing γ-secretase complexes over PSEN2-containing complexes, including the arylsulfonamides **9** (or ELN-318463) from Elan and **10** (or BMS-299,897) from Bristol-Myers Squibb (Fig. 8) (Zhao et al., 2008). Through the generation of PSEN1/PSEN2 chimeras and point mutations, specific residues (Leu172, Thr281, and Leu282) in PSEN1 were identified as necessary for the selective inhibition. Although PSEN1 appears to account for ~80% of total Aβ production (De Strooper et al., 1998), knockout of PSEN1 is perinatal lethal (Shen et al., 1997; Wong et al., 1997), and targeting PSEN1 selectively is not expected to prevent the toxic effects due to inhibition of Notch signaling. Mice deficient in PSEN2, however, are viable and fertile and develop only mild pulmonary fibrosis and hemorrhage with age (Herreman et al., 1999), and the 20% Aβ production remaining in PSEN1 deficient mice has been attributed to PSEN2 (De Strooper et al., 1998). A 20% reduction in brain Aβ may be sufficient for therapeutic purposes, and targeting PSEN2 selectively over PSEN1 could be worthwhile

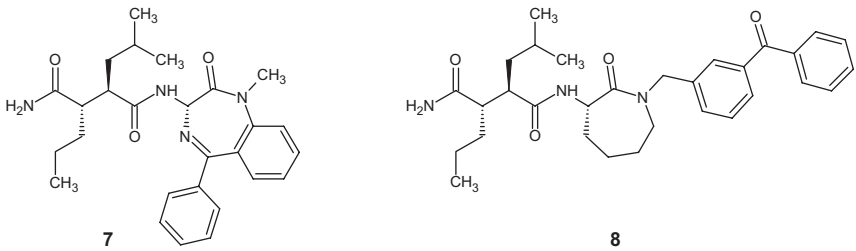


FIGURE 7 Malonamide inhibitors of γ-secretase.

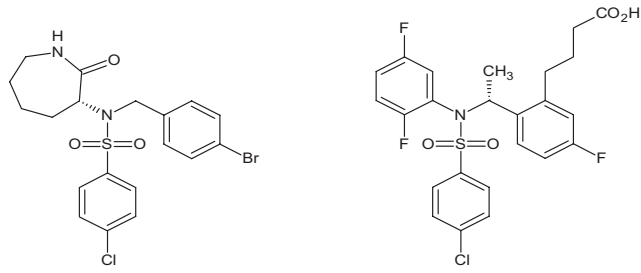


FIGURE 8 PSEN1-selective γ-secretase inhibitors.

and may be possible. PSEN2 knockout mice, however, did not display any effect on APP processing (Herreman et al., 1999), although this may be due to compensation during development. Thus, at present there is conflicting evidence regarding whether selective inhibition of PSEN2-containing γ-secretase complexes would lower Aβ levels in the brain and do so effectively enough to prevent Aβ aggregation and neurotoxicity.

VI. Modulators

Nonselective GSIs may have apparently insurmountable liabilities as AD therapeutic agents. Therefore, extensive efforts have been expended toward identifying γ-secretase modulators (GSMs) with more subtle effects on the activity of the protease. In general, modulators described to date fall into two main categories. The first are compounds that do not change the production of total Aβ or AICD but rather shift the spectrum of produced Aβ peptides toward shorter forms that are more soluble and less pathogenic. Specifically, these compounds lower Aβ42 levels and elevate Aβ37-39. Such compounds appear to have similar effects on the processing of the Notch receptor by γ-secretase; however, the release of the signaling molecule NICD is not inhibited, and toxic effects due to interference with Notch function are not observed. Compounds that alter Aβ production in this way are what are typically meant by the term γ-secretase modulator, or GSM, in the literature, even though other types of modulation are possible. The second type of modulator inhibits all cleavage of APP by γ-secretase, thereby blocking the production of all Aβ peptides, while allowing Notch proteolysis to continue, at least within a certain range of concentrations. Such compounds are typically referred to as Notch-sparing GSIs. In this review, both categories are considered GSMs, adjusting the activity as opposed to broadly inhibiting it. These two categories are denoted here as Aβ42-lowering GSMs and Notch-sparing GSMs.

A. Aβ42-Lowering GSMs

The first type of Aβ42-lowering GSM to be reported was a subset of nonsteroidal anti-inflammatory drug, or NSAIDs (Weggen et al., 2001). These included ibuprofen, sulindac sulfide, and indomethacin but not naproxen or aspirin. The ability of these compounds to lower Aβ42 in cells lacking cyclooxygenase demonstrated that the mechanism of action does not involve this common NSAID target. In parallel with the reduction of Aβ42, the compounds also elevated Aβ38, suggesting a precursor–product relationship between these two Aβ peptides that has since been demonstrated as correct by the identification of the tetrapeptide byproduct by mass spectrometry from cell-free γ-secretase assays (Takami et al., 2009).

Subsequently, the *R* enantiomer of flurbiprofen, compound **11** (Fig. 9), was found to be an Aβ42-lowering agent (Eriksen et al., 2003). As this enantiomer of a known drug is inactive toward cyclooxygenase and showed promising effects in APP transgenic mice (Kukar et al., 2007), **11** (also called Flurizan) entered into clinical trials for the treatment of AD, ultimately failing in phase III (Green et al., 2009) for reasons likely related to lack of potency and poor brain penetration. As for the molecular mechanism of this class of compounds, evidence suggests a direct effect on γ-secretase cleavage of APP (Weggen et al., 2003). The APP substrate C99 itself was identified as a target (Kukar et al., 2008), although other studies implicate the γ-secretase complex, particularly presenilin (Beher et al., 2004; Sato et al., 2006).

An arylacetic acid related to NSAIDs, compound **12** (or CHF5074) (Fig. 9) from Chiesi Farmaceutici, has been reported as an Aβ42-lowering agent that does not inhibit cyclooxygenase (Peretto et al., 2005). Although the potency of this compound is weak (IC_{50} of 41 μM for inhibition of cellular Aβ42 production), **12** lowered Aβ plaque burden, restored hippocampal neurogenesis, and reversed learning and memory deficits in APP transgenic mice (Imbimbo et al., 2007, 2009, 2010). Another arylacetic acid-type compound, **13** (or JNJ-40418677) (Fig. 9) from Johnson & Johnson and Jansen, was also found to safely reduce Aβ plaque burden upon

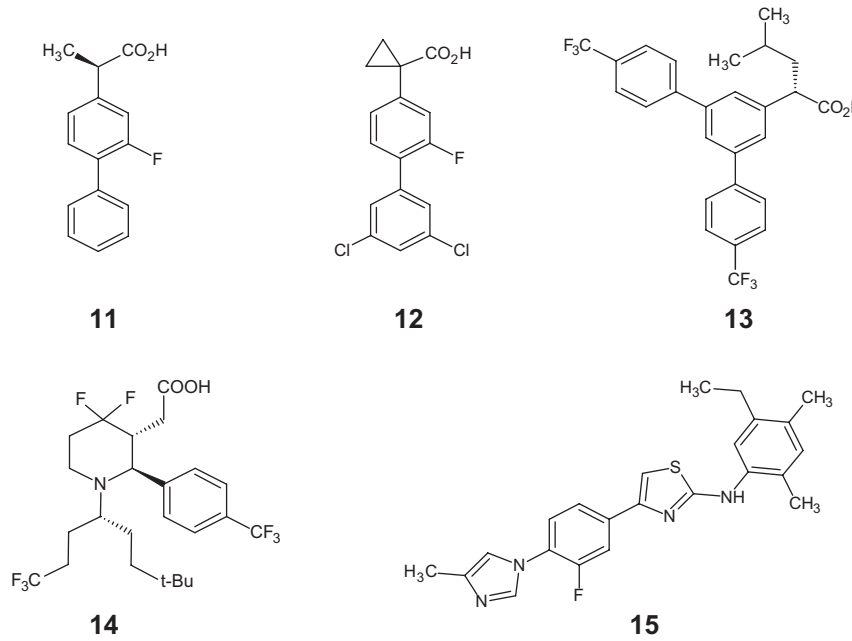


FIGURE 9 Aβ42-lowering GSMs.

chronic treatment in APP transgenic mice (Van Broeck et al., 2011). Piperidine acetic acids are another interesting class of Aβ-lowering GSM that are structurally related to but distinct from the arylacetic acids. Potencies approaching 200 nM for lowering Aβ42 in cell-based assays have been reported, and some of these compounds, exemplified by **14**, can apparently get into the brain and reduce Aβ42 levels in rodents (Stanton et al., 2009). Moreover, conversion of piperidine acetic acids into photoaffinity reagents led to the identification of presenilin as the direct target (Crump et al., 2011; Ohki et al., 2011).

A high-throughput screening campaign followed by structure-activity optimization led to the discovery of a completely different structural class of Aβ42-lowering GSMs, 2-aminothiazoles that are exemplified by **15** (Fig. 9) (Kounnas et al., 2010). These compounds are not only structurally distinct from the Aβ42-lowering NSAIDs, but they also appear to work by a somewhat different mechanism and putatively through a different target within γ-secretase. Compounds such as **15** inhibit the production of both Aβ40 and Aβ42 without affecting total Aβ levels. In parallel, Aβ37 and Aβ38 are elevated. The potency of these agents are much higher than what is seen with any NSAID-like compounds, with IC_{50}s as low as 5 nM for lowering Aβ42 in cell-based assays. The compounds could also inhibit Aβ40 and Aβ42 production in a cell-free assay, suggesting a direct effect on γ-secretase processing of APP.

Immobilization of one of these compounds to create an affinity matrix led to the identification of PSEN1 NTF, PSEN1 CTF, and Pen-2 from detergent-solubilized cellular extracts (Kounnas et al., 2010). Pen-2 was isolated quantitatively, suggesting that this small 10-kDa membrane protein component of γ-secretase is the direct target. However, as one of the detergents used for the affinity chromatography (Triton X-100) is known to completely dissociate the γ-secretase complex (Esler, Kimberly et al., 2002) and no competition with free inhibitor was tested, the possibility of a nonspecific interaction cannot be ruled out. Regardless of the exact mechanism though, oral administration of **15** lowered brain Aβ42 levels in APP transgenic mice and chronic daily dosing over 7 months substantially reduced Aβ deposition and was well tolerated, with no Notch-related toxicity observed.

B. Notch-Sparing GSMs

Although inhibition of Notch signaling was identified as a potential problem for GSIs for AD in 1999, it was unclear if selective inhibition of APP processing over that of Notch was possible. Theoretically, the enzyme could possess a binding pocket for small molecules that allosterically regulate substrate selectivity, but whether such a site might actually exist was completely unknown. Isocoumarins were initially identified as selective

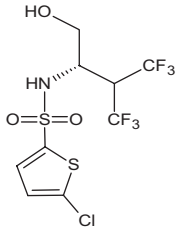
inhibitors of Aβ production from C99 that did not affect processing of Notch (Petit et al., 2001). However, these compounds were not effective in cell-free assays (Esler, Das et al., 2002), and the direct target and affected cellular pathways remain unknown. Paul Greengard's laboratory then found that the Abl kinase inhibitor Gleevec (imatinib) could inhibit γ-secretase processing of C99 to Aβ with no effect on Notch processing (Netzer et al., 2003). This selective effect was also observed in Abl kinase knockout cells, indicating another target mediated the Aβ-lowering effect of Gleevec. Certain other compounds with kinase-inhibitor scaffolds could do the same.

A subsequent study showed that ATP and other nucleotides could increase the ability of purified γ-secretase to cleave APP substrate without affecting the processing of a Notch substrate (Fraering et al., 2005). The nonhydrolyzable ATP-γS had the same effect, demonstrating that ATPase or kinase activities were not involved. Moreover, certain compounds from a library of commercially available kinase inhibitors could block the proteolysis of purified recombinant APP substrate and purified enzyme without inhibiting the cleavage of a purified recombinant Notch substrate, demonstrating that the compounds work by interacting directly with the enzyme, the substrate, or both. An ATP photoaffinity probe labeled PSEN1 CTF, which could be blocked by ATP and the APP-selective inhibitors. Altogether, these results suggested that γ-secretase contains an allosteric site for small molecules that could selectively alter APP processing over that of Notch. The role of Gleevec *per se*, however, remained unclear, as pure Gleevec had no effect in the purified enzyme assay. The Greengard lab recently reported the identification of a γ-secretase-activating protein (GSAP) as the direct target of Gleevec via affinity labeling, and substantial evidence supported the ability of GSAP to regulate APP proteolysis by γ-secretase but not Notch, both in cells and in cell-free assays (He et al., 2010). Knockdown of GSAP in an APP transgenic mouse model reduced Aβ levels and plaque formation without apparent Notch-related toxic effects, suggesting that GSAP may be a worthwhile target for AD drug discovery. However, confirmation of GSAP, both as a Gleevec target and as a *bona fide* γ-secretase activating protein, from another lab has not yet been reported.

More recently, drug discovery efforts have identified Notch-sparing GSMs with better potencies and CNS drug-like characteristics. The thiophene-containing sulfonamide **16** (GSI-953, or begacestat) (Fig. 10) was reported by researchers at what was then Wyeth (now part of Pfizer) to potently inhibit cellular production of Aβ by γ-secretase with an IC_{50} of 15 nM, while inhibition of Notch signaling was 14-fold less effective (Kreft et al., 2008; Mayer et al., 2008). Note that these two assays are quite different, so the meaning of the 14-fold selectivity is unclear. This compound was also more metabolically stable than earlier prototypes developed at

Wyeth and showed *in vivo* efficacy in an APP transgenic mouse model, reducing Aβ40 and Aβ42 in the brain by 37% and 25%, respectively, 4 h after a 5 mg/kg oral dose (Martone et al., 2009). Compound **16** was also able to reverse contextual fear conditioning deficits in these mice. Lack of Notch-related toxic side effects encouraged moving forward with this compound in clinical trials, and single-dose administration in healthy volunteers produced a dose-dependent decrease in plasma Aβ levels (Martone et al., 2009). It remains unclear if the APP/Notch selectivity of this compound will be sufficient, as several previous GSIs could chronically lower brain Aβ levels in animal models without apparent Notch-related side effects. In general, a therapeutic window may be more readily identified in a genetically homogeneous animal population maintained in the same environment than in a heterogeneous population of AD patients living in a variety of environments.

Bristol-Myers Squibb has also reported the Notch-sparing GSM **17** (BMS-708163) (Fig. 10), which has advanced into clinical trials. Like the Wyeth compound, **17** is an arylsulfonamide, but this oxadiazole-substituted analogue is considerably more potent, with an IC_{50} of 0.30 nM for inhibiting



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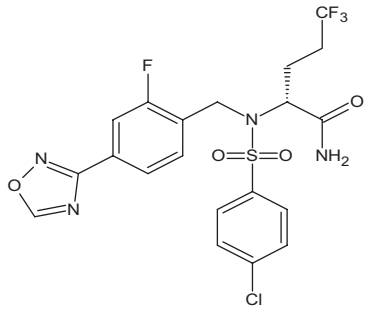
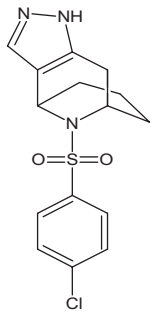
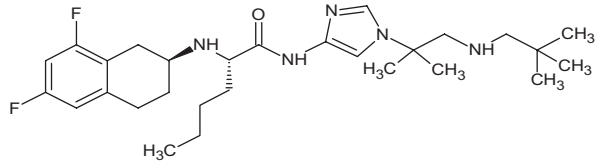


17



18



19

FIGURE 10 Notch-sparing GSMs.

cellular Aβ production, and more selective with respect to Notch, with an apparent selectivity of 193-fold (Gillman et al., 2010). As noted above for **16**, the meaning of the selectivity index is unclear, as the APP and Notch processing assays were quite different, with the former measuring Aβ and the latter measuring a reporter signal. Compound **17** showed good pharmacokinetic properties in rats and dogs and also lowered brain and CSF Aβ40 in both species at 1–2 mg/kg oral doses. Notably, chronic dosing at 10 times the concentration required for lowering Aβ did not cause Notch-related toxic effects. The correlation between brain and CSF Aβ40 lowering activity in dogs suggested that CSF Aβ40 may serve as a surrogate biomarker for brain Aβ40 levels in humans. Compound **17** also lowered CSF Aβ40 and Aβ42 levels in healthy human volunteers with dosing up to 28 days.

Elan has also reported novel arylsulfonamides as Notch-sparing GSMs, exemplified by **9** (ELN318463) (Fig. 8) and **18** (ELN475516) (Fig. 10) (Basi et al., 2010). These compounds have been reported to display 120- and 82-fold selectivity, respectively, for inhibiting Aβ production in cells compared to inhibiting Notch signaling. Again, the differences between the assays (Aβ measurement vs. signaling reporter) may make the selectivity seem higher than what would be seen in more comparable assays. Indeed, the nonselective transition-state analogue inhibitor **2** showed 14-fold selectively in these cellular assays, and enzyme assays for APP and Notch substrates, in which the products were both measured by ELISA, revealed 51- and 14.5-fold selectivity for **9** and **18**, respectively. *In vivo* lowering of brain Aβ in mice was observed after 7 days of dosing with **18** without overt signs of toxicity, although 1 week is likely not long enough to reveal Notch-related effects. Another Elan sulfonamide, ELND-006, which has advanced into clinical trials, has been reported to have a similar selectivity profile to **18**. Whether this level of selectivity is sufficient for lower CSF Aβ in human without Notch-related toxicity upon chronic exposure remains to be seen.

Pfizer has also developed a Notch-sparing GSM **19**, although this compound, PF-3084014 (Fig. 10), is not an arylsulfonamide but rather a novel tetralin imidazole (Lanz et al., 2010). This compound potently inhibited Aβ production in cells, with an IC_{50} of 1.2 nM, but inhibited Notch-dependent maturation of B- and T-lymphocytes in a fetal mouse thymus organ culture with IC_{50}s of 1–3 μM. Again, comparing these two assays may not be appropriate, and so it is difficult to know what to make of the 1000–3000-fold APP/Notch selectivity of PF-3084014. As a benchmark, the relatively nonselective compound **5** showed an IC_{50} of 21 pM for lowering cellular Aβ production and a mean IC_{50} in the fetal thymus organ culture of 4 nM, a nearly 200-fold difference. Acute treatment in guinea pigs showed some selectivity for reducing brain Aβ40 over the more aggregation-prone Aβ42. Of further concern was the apparent elevation of Aβ43 levels. This longer Aβ variant has been recently reported to lead to cerebral plaque formation and neurotoxicity in APP/PSEN1 double transgenic mice (Saito et al., 2011)

and may play an important role in AD pathogenesis. As **19** was administered subcutaneously or by osmotic pump to mice and guinea pigs, the oral bioavailability of this compound is unclear.

VII. Conclusion

γ-Secretase remains a target of keen interest for the potential prevention or treatment of AD. The focus, however, has clearly shifted toward modulators that minimize effects on Notch signaling function, with compounds that either shift the site of γ-secretase cleavage to produce shorter forms of Aβ or those that selectively inhibit APP processing by γ-secretase while allowing the enzyme to continue processing Notch. Inhibitors and modulators have also served as important research tools for the identification of the enzyme complex and probes for the topology of the active site, the substrate docking site, and allosteric binding pockets. Present compounds under investigation may not have sufficient potency, brain penetration, or selectivity to effectively lower brain Aβ while avoiding Notch-related toxicity.

Key questions remain: Is there a ceiling on the achievable APP/Notch selectivity of a GSM? If interference with Notch function can be avoided, will other toxic effects be revealed due to inhibition of intramembrane proteolysis of other γ-secretase substrates? Where are the allosteric binding sites on the γ-secretase complex with which GSMs interact? What are the topographies of these sites, and can this knowledge be leveraged for structure-based design? Does substrate contribute to the binding site of GSMs? Answering these questions should facilitate the development of optimal agents that would help provide the final test for the amyloid hypothesis—the prevention or treatment of AD by safely blocking Aβ production in the brain.

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Abbreviations

Aβ	amyloid β-protein
Aib	aminoisobutyric acid
AICD	APP intracellular domain
APP	amyloid β-protein precursor

CTF	C-terminal fragment
C83 and C99	APP CTFs produced by α- and β-secretase (respectively)
GSI	γ-secretase inhibitor
GSM	γ-secretase modulator
NICD	Notch intracellular domain
NTF	N-terminal fragment
PSEN1	presenilin-1
PSEN2	presenilin-2

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Altering Mitochondrial Dysfunction as an Approach to Treating Alzheimer's Disease

Abstract

Mitochondrial dysfunction appears to be a precipitating or exacerbating factor in both familial and late stage Alzheimer's disease. This chapter summarizes various mechanisms by which dysfunction of mitochondrial metabolism can be involved in loss of cognitive function as well as in the exacerbation of structural changes in the signature pathology of Alzheimer's disease. Although currently few in number, a number of mitochondrially directed/metabolic approaches are now being tried that include limiting the damage caused by dysfunctional oxidative metabolism. There is a clear need to identify and test specific targets to take advantage of a growing understanding in this field. The eventual successful approach to meaningfully treat Alzheimer's disease will likely include treatments aimed at correction of the mitochondrial dysfunction component.

I. Introduction

Alzheimer's disease is the most common form of dementia; it has no cure, and the number of people with Alzheimer's will be more than double over the next several decades. Considerable evidence has emerged connecting dysfunctional changes in mitochondrial metabolism to the pathology of Alzheimer's disease. However, as the definition of the disease includes the

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observance of two key pathological changes observed on autopsy, namely amyloid plaques and neurofibrillary tangles composed of hyperphosphorylated microtubule-associated protein tau, most efforts to develop pharmacological tools that might arrest the course of the disease have directly targeted the biochemistry around these phenomena (Roberson & Mucke, 2006; Goedert & Spillantini, 2006). Strong support for amyloid-related approaches comes from evidence from the familial, early onset, form of the disease, which show that several mutations in the production or processing of β-amyloid are in fact associated with increased risk of the disease (Selkoe, 2001). As detailed elsewhere in this series, these approaches have thus far resulted in limited success. This suggests a consideration should be given to the importance of the decline in mitochondrial metabolism as an additional target for therapeutic intervention.

Here, we discuss how the natural history of Alzheimer's disease includes in the earliest point of measurement of cognitive decline, a reduction in mitochondrial function. This decline in mitochondrial function may well be the factor that provides the connection of the disease with aging such that there is a seemingly inexorable increase in incidence as the population ages (Hebert et al., 2010). One can imagine that this would be the case whether the decline in mitochondrial function was the precipitating factor for an individual case of Alzheimer's disease and cognitive decline or whether the decline in mitochondrial function merely worked in concert with other pathologies relating to amyloid, microtubule function, or lipid metabolism. Given these complexities, considerable heterogeneity must exist amongst various cases. Nonetheless, there is evidence that decline in mitochondrial function is occurring as part of the natural history of the disease. This decline in mitochondrial function can be exacerbated by other factors known to be involved in the Alzheimer's pathology, and, moreover, decline in mitochondrial function creates a viscous cycle of pathologies by reducing repair mechanisms. This information provides a framework for logical approaches that could affect the progress of Alzheimer's disease by targeting mitochondrial or metabolic processes.

II. Theories of Pathogenesis and the Natural History of Alzheimer's Disease

Hodges has reviewed how careful studies by many investigators have now shown that there is clearly a progressive loss of cognitive function that proceeds and predicts the devastating dementia (Hodges, 2006). Armed with this knowledge, it has recently become possible to follow the disease longitudinally to gain further insight into the etiology that should help in the decisions of who and how to treat. In 2004, a consortium of government and private entities initiated the AD Neuroimaging Initiative (ADNI). This

initiative supports the measurements of many biomarkers to go along with the measurement of specific cognitive function, but one of the most sensitive and most effective at detection of early changes is the decline in brain glucose metabolism measured by ^{18}F-2- deoxyfluo glucose (FDG) (Fukuyama et al., 1994; Langbaum et al., 2009; Jagust et al., 2010). Hypometabolism may be a general function of cognitive decline (Blass, 2001), but more particularly there is a shift in brain metabolism in Alzheimer's disease (Yao, Rettberg, Klosinski, & Cadenas, 2011). Numerous studies have shown that regional declines in brain glucose metabolism has been detected by positron emission tomography (PET) imaging with FDG in late onset Alzheimer's (e.g., deLeon, et al., 2001, Forquet et al., 2009; Chen et al., 2010), but this also appears to be the case even in early-onset familiar cases (Mosconi et al., 2006). Thus, evidence indicates that a decline in glucose metabolism precedes the other findings from loss of cognitive function to atrophy of brain tissue. The cause of the hypometabolism might well lie with defects in mitochondrial function.

The evidence of mitochondrial dysfunction in Alzheimer's diseases has been well chronicled (e.g., Hirai et al., 2001; Zhu et al., 2004; Parihar & Brewer, 2006; Ankarcrona et al. 2010). Many studies have reported a decline in key mitochondrial enzymes, especially pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, two enzymes that regulate the flow of carbon through the Kreb's cycle. Interestingly, both of these complexes are dependent on lipoic acid in their reaction mechanism and they are both exquisitely sensitive to inhibition and destruction by reactive oxygen. Another enzyme that is extremely sensitive to damage by reactive oxygen is cytochrome oxidase, the terminal enzyme of the respiratory chain, and it is also reported to be decreased in the brains of Alzheimer's patients and in animal models. These data would be consistent with progressive mishaps in oxidative metabolism, which by generating reactive oxygen would further reduce metabolism by inactivation of these key rate-limiting steps. The generation of reactive species and metabolic dysfunction might lead to progressive damage to mitochondrial regulatory systems and these cumulative effects could form the basis for the progressive impact of aging on all of these processes including progressive mutations in mitochondrial DNA in various tissues (Wallace, 2008). Cumulative damage to the mitochondria not only results in hypometabolism, reducing energy for important cell functions including maintenance of compartmentalized membrane potential and cell-to-cell communication and including synapses, but would also interfere with coordinated removal of misfolded proteins and dysfunctional organelles. In the extreme case this would lead to cell death and atrophy; however, in the absence of observed atrophy there would be progressive decline in function and the build-up of structural pathologies, especially in cases where there is an additional issue with production or metabolism of amyloid precursor protein (APP), for example. Whether or not there is an inborn error in the metabolism of key proteins such as APP or tau,

progressive changes can occur with aging and together this could result in the progressive failure to result in late onset Alzheimer's (Pasihar and Brewer, 2006). As discussed in the following sections, structural pathologies can lead to effects on mitochondrial function and changes in mitochondrial function can prevent corrections of structural pathologies leading to a progressive decline.

Thus, there is overwhelming evidence that mitochondrial dysfunction occurs in Alzheimer's and that it most likely is contributing either directly or indirectly to the pathology.

III. Other Pathologies Can Affect Mitochondrial Function

Several proteins that are associated with AD risk or disease pathogenesis have been shown to influence mitochondrial function. These include the APP and some of its proteolytic cleavage products including Aβ1-42 (beta amyloid, Aβ), the microtubule associated protein (MAP) tau, and the cholesterol trafficking protein ApoE4, the isoform that significantly increases the risk of developing AD. In this section, we highlight some of the more well-characterized interactions of these three proteins with mitochondria. More comprehensive reviews of this topic can be found in several recent reviews (Tillement et al., 2011; Muller et al., 2010; Swerdlow et al., 2010; Swerdlow 2011; Reddy 2011).

A. Amyloid Precursor Protein and Beta-amyloid

Both the APP and Aβ accumulate in mitochondrial membranes, as well as in the mitochondrial import channels leading to structural and functional damage (Pagani & Eckert, 2011; Tillement et al., 2011). The *N*-terminal portion of APP contains three positively charged residues that are similar to mitochondrial targeting signals found in p450 cytochromes, which targets APP to mitochondria in human cortical HCN1a neurons and in brains of Tg2576 mice (Anandatheerthavarada et al., 2003). The APP is only partially inside the mitochondria, since trypsin releases about a 73 kD portion that is exposed. Moreover, the accumulation of APP in a transmembrane orientation was associated with a reduction in mitochondrial membrane potential and ATP levels indicating that APP damaged the mitochondria and impaired energy metabolism. In samples prepared from AD brains, APP was found associated with TOM40 (translocase of the outer mitochondrial membrane) and TIM23 (translocase of the inner mitochondrial membrane) (Devi et al., 2006). The accumulation of APP in these import channels reduced import of nuclear encoded proteins that are normally targeted to the mitochondria, including cytochrome c oxidase subunits IV and Vb. The levels of mitochondrial APP were associated with reduced cytochrome c

oxidase activity and increased levels of hydrogen peroxide, and were directly correlated with mitochondrial dysfunction measured in different brain regions of AD patients.

Evidence that Aβ can accumulate in mitochondria includes studies showing that the mitochondrial protein β-amyloid binding alcohol dehydrogenase (ABAD), a protein involved in detoxifying aldehydes produced by oxidative stress, directly interacts with Aβ within the mitochondria (Lustbader et al., 2004). The ability of ABAD to detoxify aldehydes such as 4-hydroxy-2-nonenal (4HNE) is inhibited by Aβ, which reduces its cytoprotective actions against reactive oxygen species (ROS) (MurakamiOhsawa et al., 2009). Additional evidence for the presence of Aβ in mitochondria comes from Western blot analysis of mitochondria isolated from the cortex of Tg2576 transgenic and wildtype mice (Manczak et al., 2006), which showed the presence of both Aβ1-40 and Ab1-42 in the Tg2576 mice. Similar findings were observed in mitochondria isolated from mouse neuronal N2a cells overexpressing mutant APP. Further fractionation using digitonin allowed localization of Aβ to the mitoplast (the inner membrane and matrix). These authors further showed that mitochondria from Tg2576 mice had higher levels of hydrogen peroxide and of protein carbonyls, and decreased levels of cytochrome c oxidase. Using rat liver mitochondria, it was shown that Aβ is transported into the mitochondria via the TOM import complex and accumulates within the cristae, and that this uptake is not dependent upon the membrane potential (Hansson Petersen et al., 2008).

Another source of mitochondrial Aβ is via cleavage of APP within mitochondria by γ-secretase. The γ-secretase complex contains presenilin-1 (PS1), nicastrin (NCT), APH-1, and PEN-2. As observed for APP, NCT (but not PS1, APH-1, or PEN-2) contains a mitochondrial targeting sequence and can be observed within brain mitochondria by immunoelectron microscopy (Hansson et al., 2004). Further, NCT was found in the mitochondria in a high molecular weight complex containing the other three γ-secretase proteins, suggesting that a preformed cytosolic γ-secretase complex is transported to the mitochondria. The mitochondria γ-secretase is active, since incubation of isolated mitochondria with the APP C-terminal fragment generated APP intracellular domain whose production was inhibited by γ-secretase inhibitors (Pavlov et al., 2011). Since β-secretase has not been described within mitochondria, the production of Aβ may be due to beta-secretase1 (BACE1) cleavage of APP in the cytosol, which generates a substrate for the mitochondrially located γ-secretase.

Intramitochondrial Aβ can interact with lipid components as well as protein. A biophysical analysis using artificial unilamellar vesicles provides evidence that Aβ influences mitochondrial morphology and function by reducing the ability of the inner mitochondrial membrane to form or maintain cristae during local changes in pH; and is due to an Aβ-dependent

dehydration of the lipid bilayer, loss of membrane fluidity, and changes in the interactions between the two membrane surfaces (Khalifat et al., 2012).

Once inside mitochondria, Aβ may not be cleared as efficient as cytosolic Aβ. Both cytosolic and extracellular Ab can be reduced by metalloproteases such as neprilysin and insulin degrading enzyme (IDE). Similarly, mitochondrially located Aβ can be degraded by the enzyme presequence protease (PreP) that is localized in the matrix and is an analogue of IDE (Alikhani et al., 2009). However, PreP's activity is reduced in AD brains as compared to nonAD controls, which may contribute to Aβ accumulation (Alikhani et al., 2011).

The accumulation of Aβ in mitochondria is not a homogeneous phenomenon, but shows differences depending upon subcellular locale. In mutant APP transgenic J-20 mice, the synaptically located mitochondria, necessary to provide ATP for synaptic transmission, showed greater Aβ accumulation, and increased mitochondrial dysfunction than did nonsynaptically located mitochondria (Du et al., 2010). The data suggest that synaptically located mitochondria are more sensitive to Aβ accumulation, consistent with findings in AD patients and mouse models that these mitochondria undergo dysfunctions earlier than nonsynaptic located mitochondria (Du et al., 2011).

Other cleavage products of APP have been shown to interact with mitochondria. Using a combination of immunostaining and digitonin fractionation methods, the C-terminal fragment (C99) produced upon β-secretase cleavage of APP was shown to accumulate in brain mitochondria isolated from transgenic 5xFAD mice, as was the full length APP (Devi & Ohno, 2012). Interestingly, depletion of BACE1 not only reduced targeting of C99, but also prevented targeting of the full-length protein, suggesting that APP uptake may be dependent upon the activity of, or association with the BACE1 proteins.

There are several important consequences of Aβ and APP accumulation within mitochondria. Aβ induces mitochondrial fragmentation and reduces mobility (Leuner et al., 2012), and is associated with increases in mitochondrial fission factors (Wang et al., 2008) and reduced axonal transport of mitochondria (Calkins & Reddy, 2011;Wang, Perry, Smith, Zhu, 2010;). Mitochondrial fission is regulated by GTPases including dynamin-related protein 1 (Drp1) that is primarily cytosolic but also associates with the outer mitochondrial membrane (Chen & Chan, 2009). Levels of Drp1, as well as of other proteins involved in fission, were found to be increased in AD brains, as were interactions of Aβ monomers and oligomers, suggesting that increased mitochondrial fission is mediated by increased Drp1 (Manczak et al., 2011).

Several studies have shown that APP can reduce mitochondrial function. In human HEK293 cells, overexpression of either human wildtype APP or the Swedish mutation APPsw decreased the mitochondrial membrane potential, inhibited complex I activity, and decreased ATP levels

(Hauptmann et al. 2009; Leuner et al., 2012; Rhein et al., 2009a). Aβ directly inhibits other mitochondrial proteins, including cyclooxygenase IV (COX IV) (Crouch et al., 2005), and the ATPase α-subunit, thereby reducing ATP synthesis (Schmidt et al., 2008).

Aβ can also interact with protein components of the mitochondrial permeability transition pore (MPTP) (Singh, Suman, Chandna, & Das, 2009), leading to changes in permeability (Moreira et al., 2002). Overexpression of APP induces a reduced glutathione (GSH)-sensitive opening of the MPTP, which leads to cytochrome C release and induction of apoptosis (Bartley et al., 2011).

In human embryonic kidney 293 cells, inhibition of complex I with rotenone, which increases superoxide production, led to a significant increase in Aβ1-40 levels at 2h treatment (Leuner et al., 2012). Inhibition of complex III with antimycin also increased Aβ 1-40 levels. There was also a concomitant increase in BACE1 activity, which could account for the increased generation of Aβ1-40. The same authors showed that in mice with a complex I deficiency, due to a mutation in complex I (the Ndufs4 gene), soluble brain levels of Aβ 1-40 are increased compared to wildtype mice. There were also increased Aβ1-40 levels in brain extracts following treatment of transgenic mice (human Swedish and London mutations in APP) when treated with rotenone for 3 days. Since Aβ induces mitochondrial dysfunction and ROS increases, these findings point to a positive feedback loop leading to increased metabolic loss and increased amyloid accumulation. A summary of the ways in which APP or fragments of APP can interfere with mitochondrial function is shown in Fig. 1.

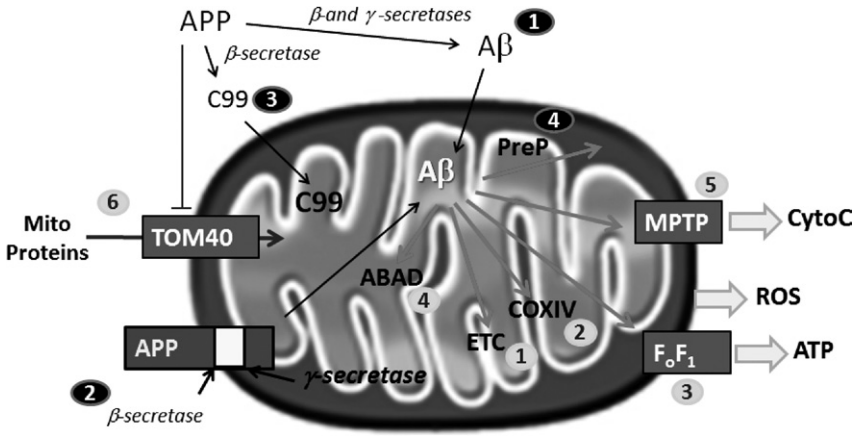

FIGURE 1 Effects of APP and related molecules on mitochondrial function. APP and related fragments can accumulate in mitochondria by the following means: (1) direct accumulation of Aβ into cristae; (2) cleavage of APP by cytosolic β-secretase and mitochondrial γ-secretase; (3) direct accumulation of 99 residue C-terminal fragment; and (4) loss of intramitochondrial cleavage by the PreP protease.

APP and related fragments can directly affect mitochondrial function by the following: (1) inhibition of electron transport chain complexes, (2) inhibition of COX IV, (3) direct inhibition of ATP synthase activity, (4) inhibition of β-ABAD generating ROS, (5) induction of cytochrome C release through the MTTP, and (6) blocking protein transport through TOM40 importer.

B. Tau Protein

General reviews of tau and its role in AD have been published recently (Lee et al., 2011; Pritchard et al., 2011; Iqbal et al., 2010). In brief, tau is the major MAP in mature neurons. MAPs including tau interact with tubulin and promote formation and stabilization of microtubules, which are dependent upon the phosphorylation state of the MAP. The human tau protein exists in six different isoforms, and the longest form contains 79 potential phosphorylation sites on serine and threonine residues. Tau is normally phosphorylated on an average of 2–3 phosphoryl groups per molecule, but that is increased by 3- to 4-fold in AD which reduces the association of tau with microtubules. The hyperphosphorylated tau can aggregate to form paired helical filaments which intermix with straight filaments to form neurofibrillary tangles.

Studies of the effects of tau protein on mitochondria are more limited than those of the APP or Aβ. Tau overexpression *in vivo* led to a progressive disruption in mitochondrial redistribution with age (Kopeikina et al., 2011). Tau protein can impede mitochondrial axonal transport (Stoothoff et al., 2009). Tau is cleaved at Asp 421 producing a fragment that induces mitochondrial fragmentation (Quintanilla et al., 2009). Interestingly, synergistic actions of Aβ and tau on mitochondrial function have been described (Eckert et al., 2010; Rhein et al., 2009b; Rhein & Eckert 2007). The *N*-terminal fragment of tau interacts with mitochondrially located Aβ together with the mitochondrial adenine nucleotide translocater-1 (ANT1) and cyclophilin D, leading to inhibition of nucleotide exchange (Amadoro et al., 2011). Similarly, cleaved tau alone impairs mitochondrial function in neurons, and further increases in oxidative stress occur when that is combined with low, sublethal concentration of Aβ (Quintanilla et al., 2012).

Since mitochondrial stress can increase tau phosphorylation (Melov et al., 2007), tau-induced mitochondrial damage similarly creates a cycle leading to significant mitochondrial impairment.

C. Apolipoprotein E

Apolipoprotein E (ApoE), involved in lipid handling, has three major isoforms with ApoE4 being a major risk factor for AD. ApoE4 is present

in 40–65% of the AD cases, and both increases risk and lowers the age of onset. ApoE is a lipid acceptor protein, and is involved in cholesterol transport and formation of high-density lipoproteins (HDL) which are needed for neuronal growth and synaptogenesis. The ApoE4 protein has been characterized in numerous ways to help explain the mechanisms underlying its damaging actions, with most investigators focusing on examination of ApoE4's effects on amyloid accumulation and clearance. Several recent reviews of ApoE in AD have been published (Huang, 2010; Verghese et al., 2011). Of interest to the current discussion, several studies have shown that mitochondrial function can be influenced by ApoE isoforms (Reddy 2011).

ApoE can be readily cleaved by a serine protease that is expressed at high levels in AD brains (Harris et al., 2003). This protease generates a C-terminal fragment that is neurotoxic (Huang et al., 2001), and the ApoE4 allele is cleaved at greater efficiency than the other ApoE alleles. In neuronal N2a cells, the C-terminal ApoE4 fragment (1–272) was neurotoxic, although the full-length protein was not, suggesting the *N*-terminal region may have protective functions. This C-terminal fragment form inclusions within mitochondria containing phosphorylated tau, which may contribute to mitochondrial dysfunction. The C-terminal fragment also contains the lipid binding region (residues 241–272) and mutations in this region prevented neurotoxicity, while deletion or mutation of residues within the receptor binding regions (AA 135–150) abolished interactions with mitochondria (Chang et al., 2005). Since ApoE4 can also be cleaved by endogenous proteases, formation of various C-terminal fragments could contribute to mitochondrial damage in AD. The ability of ApoE4 to disrupt neuronal mitochondrial function requires the presence of Arg-61, a residue unique to ApoE4 that governs intramolecular interactions, and treatment with small molecules to disrupt those interactions prevents mitochondrial damage (Chen et al., 2011).

ApoE4 associates with a large number of mitochondrial proteins. Using immunochromatography methods to identify proteins in mouse brain that could associate with ApoE4, Nakamura et al. (Nakamura et al., 2009) found that of the 16 proteins identified, 10 were known to be associated with mitochondria. This included components of complex III and IV. They also showed that overexpression of ApoE4*N*-terminus inhibited both complex III and IV activities and that this was associated with a reduced mitochondrial membrane potential and lower ATP levels. A more recent proteomics approach (James et al., 2012) using transgenic mice expressing human ApoE3 or ApoE4 found that ApoE genotype significantly alters patterns of mitochondrial protein expression in the hippocampus under basal conditions, as well as in response to global ischemia; and many of these are involved in the regulation of energy production and oxidative stress.

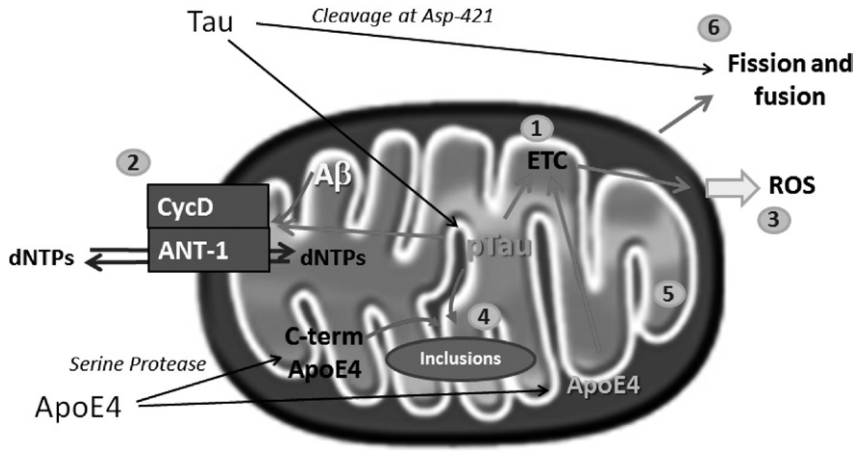

FIGURE 2 **Tau pathologies and ApoE4 inhibit mitochondrial function.** Tau and ApoE4 or fragments of ApoE4 can associate within the mitochondria in the following ways to (1) inhibit the electron transport chain; (2) directly inhibit the adenine nucleotide transporter (ANT1) blocking ATP production; (3) increase ROS production; (4) form inclusions; (5) directly interfere with mitochondrial protein assembly; and (6) affect fission and fusion, as well as mitochondrial mobility.

A summary of the mechanisms by which tau pathologies and ApoE4 can interfere with mitochondrial function is shown in Fig. 2.

IV. Mitochondrial Function Can Exacerbate Other Pathology

While specific AD pathologies can interfere with mitochondrial functions, decline in mitochondrial function impacts many cellular processes needed for maintenance of cellular function. Green et al., (2011) recently reviewed the importance of mitochondria in controlling the processes of inflammation, autophagy, and apoptosis. They propose that reduced ability to clear damaged cellular components (autophagy, or mitophagy in the case of removing dysfunctional mitochondria) leads to increased inflammation and cell death. These processes are progressively affected with aging and in Alzheimer's disease. Moreover, Green et al. review literature showing that issues with presenilin-1 itself can directly affect autophagy processes by altering lysosome function and, moreover, mutated forms of presenilin have been shown to accumulate in the specialized regions of the endoplasmic reticulum (ER) that are associated with mitochondria (Area-Gomez et al., 2009). The fact that mutated presenilin-1 has been shown to accumulate in the portions of the ER and that this seems to also be the case with other incorrectly folded proteins as well (Schon & Area-Gomez, 2010)

suggests that the mitochondria could also be influencing the metabolism and clearance of mutated proteins. Zampese et al. (2011) have shown that presenilin-2 also has a regulatory role in interactions between the ER and other cellular structures. Recently, Pavlov et al. (2011) have shown that a significant amount of γ-secretase activity against APP occurs in the mitochondria. It is important to recognize that the clearance of misfolded proteins is a response coordinated by signals beginning in the ER by a process known as the unfolded protein response. However, it is becoming increasingly clear that the mitochondria also play an important role in the regulation of this function (Malhotra & Kaufman, 2011). Thus, a decline in mitochondrial function could exacerbate issues with misfolded proteins that contribute to amyloid deposits and microtubule tangles. Treatments that improve mitochondrial function might help regulate the clearance of damaged organelles, cells, or macromolecular deposits.

Mitochondrial function requires continued production of organelles that are in a dynamic equilibrium with other cellular structures and this requires a balance of both fission, where the mitochondria become smaller, and fusion, where they join together into larger structures. These processes are out of balance in Alzheimer's disease (Wang et al., 2009) likely at least in part because of oxidative damage to some of the key regulatory machinery required to control these mechanisms (Cho et al., 2009; Manczak et al., 2011). Prevention of the decline in the overall mitochondrial function might be able to restore the normal activities that are involved in the dynamic control of these organelles.

V. Therapeutic Approaches Being Taken and Opportunities Suggested

Thus, there is a large body of evidence that consideration should be given to interventions that correct/prevent the decline of mitochondrial function in the treatment of Alzheimer's disease. One of the most obvious places to begin to prevent the dysfunction in mitochondria is the generation of ROS during oxidative metabolism. Defective metabolism is theorized to be a major component connecting metabolic disease, overnutrition, and aging to declining mitochondrial function. As discussed above, generation of excess ROS is expected to play a key role in at least part of the direct damage that occurs. Thus, preventing the ROS damage would prevent the progressive decline of mitochondrial function that occurs during the aging process. Limitation of ROS-induced damage could be accomplished by approaches that either control the production of these reactive intermediates by selectively altering metabolism of certain substrates or by scavenging the reactive molecules once they are produced. The potential advantages of such approaches are shown by the significant extension of life span in mice

that have reduced caloric intake or in mice that express catalase specifically targeted to the mitochondrion (Schriner et al., 2005). Some examples of various specific therapeutic approaches along these lines are considered below.

One mitochondrially targeted antioxidant that has recently been evaluated in animal models is MitoQ, which is a covalent construct of ubiquinone and triphenylphosphonium (TPP). As ubiquinone is a component of the electron transport mechanism, this molecular complex is incorporated into the mitochondrial matrix side of the inner mitochondrial membrane where it is reduced to ubiquinol, an active antioxidant. Apparently, since this compound is a poor substrate from complex I and complex III, the incorporation of the complex into the inner mitochondrial membrane does not function in the electron transport mechanism, but the molecular molecule remains there locally to regenerate complex II and produce a local antioxidant action (James et al., 2005, James et al., 2007).

The efficacy of MitoQ in terms of Alzheimer's disease-related pathology has been evaluated *in vitro* and in a mouse model. McManus et al., (2011) demonstrated that the coadministration of nM MitoQ with Aβ peptide to mouse cortical neurons was able to block the effects of the amyloid peptide on increasing ROS and prevent the loss of mitochondrial membrane potential. Treatment of female 3xTg-AD mice, which have defects in APP, Presenilin-1, and tau and associated mitochondrial stress, with MitoQ (given as 100 uM in drinking water) from 2 to 7 months significantly, improved learning and special memory retention to a level observed in nonmutant mice. This appeared to be a specific effect not observed with control treatments, which included unmodified TPP. Moreover, the MitoQ treatment also decreased the markers of oxidative stress in the brains of these mice, providing support for the mechanism. However, a phase 2 trial of MitoQ (40 and 80mg) in 128 patients with Parkinson's disease did not provide positive results (Snow et al., 2010) and it is not evident that clinical trials are underway with this compound for treatment of Alzheimer's disease at this time.

Although not specifically a mitochondrial target, there are data showing the regulation of mTOR may have application to the treatment of Alzheimer's. In cases of over nutrition, there is an overactivation of mTOR, which signals the need to store excess calories rather than to burn them immediately. This signaling pathway has a direct connection with mitochondrial function since mTOR activation is a potent inhibitor of mitophagy (Kim et al., 2007). As discussed above, a chronic inhibition of mitophagy results in retention of damaged organelles and limitation of the clearance of misfolded or misfunctioning components. Caccamo, Majumder, Richardson, Strong, and Oddo,(2010) demonstrated that rapamycin, a specific inhibitor of mTOR, rescued the pathology of the

3xTG-AD mice including a reduction of amyloid and tau pathology and improvement of learning and memory. This reduction in Aβ and tau pathology were not due to changes in protein production. However, these data clearly demonstrated that the rate of clearance of the structural pathology correlated with an increase in markers for autophagy. Moreover, blocking autophagy with 3-methyladenine blocked the ability of rapamycin to reduce Aβ42 levels. Thus, inhibition of mTOR increased autophagy and this increased the level of clearance of Alzheimer's pathology in the mutant mice. It is also possible to inhibit mTOR indirectly by activation of AMPK, for example, and the potential for this is also under examination (Vingtdeux, 2011). There may be multiple mechanisms by which inhibition of mTOR may reduce the level of inflammation in both neurons and glia (Lisi et al., 2011). As of yet, there do not appear to be any clinical trials underway to directly evaluate this approach in humans.

Interestingly, especially given the connection of metabolism and Alzheimer's disease, there appears to be a link between Alzheimer's disease and Type 2 diabetes. It is generally accepted that individuals with established diabetes and/or borderline diabetes have an increased risk of developing Alzheimer's disease (Arvanitakis et al., 2004; Nooyens et al., 2010). Also interestingly, several clinical trials have suggested the potential for a positive effect of the insulin sensitizers, compounds which were initially developed to treat diabetes, in Alzheimer's disease (Hanyu et al., 2009; Hanyu et al., 2010) or to prevent cognitive impairment in older individuals (Abbatecola et al., 2010). The rationale to conduct those studies was provided by preclinical studies that demonstrated that these molecules could impact inflammation, amyloid pathology, and function in rodent models of AD (e.g., Heneka et al, 2005; Nicolakakis et al, 2008; Petersen et al, 2006). However, longer-term studies with rosiglitazone in Alzheimer's were equivocal and no longer-term definitive studies have yet been conducted with pioglitazone (Miller et al., 2011). Given a new understanding of the mechanism of action of these insulin sensitizers, it may be the first generations compounds may not be the best compounds to evaluate for this use.

Although the first generation insulin sensitizers were activators of the nuclear transcription factor PPARγ, new agents in development are seeking to capitalize on a direct effect on mitochondrial function.

The fact that insulin sensitizers could have important effects on mitochondrial function has been recognized (reviewed by Feinstein et al., 2005). Importantly, it appears that, in fact, a key part of the beneficial pleiotropic pharmacology of these compounds may be related to a specific effect on mitochondria that could reduce the degree of metabolic inflammation (Colca 2006). The misconception that the agents, which were originally discovered empirically, worked solely by selective

activation of the nuclear transcription factor PPARγ may have prevented the development of new agents that take advantage of the mitochondrial interaction (Colca & Kletzien, 2006). Indeed, of the clinical agents from the first generation approved for clinical use, pioglitazone, the one with the weakest PPARγ activity is the only agent that has proven to have a sustainably beneficial clinical profile in the prolonged treatment of diabetes (Ryder, 2011). In contrast, rosiglitazone has essentially been removed from the market for all utilities. Recently, a class of insulin sensitizers optimized for the mitochondrial action are being developed and these compounds appear to affect cellular differentiation and mitochondrial biogenesis through a yet undisclosed mitochondrial target (McDonald, 2010a,b). The mechanism of action of these compounds appears to include an adjustment of nutrient sensing pathways as well as a molecular break on Wnt signaling. A clinical trial of one of these compounds, MSDC-0160, which is also under development for treatment of type 2 diabetes (Colca et al., 2009), is currently underway in patients with mild to moderate Alzheimer's disease (NCT01374438). A key component of this study will be the measurement of treatment-related changes in brain glucose metabolism as measured by PET.

VI. Conclusion

It is evident that progressive mitochondrial dysfunction contributes either directly or indirectly to the pathogenesis of Alzheimer's disease. Pathologies directly related to familial, early-onset Alzheimer's such as APP processing or tau posttranslational modifications, can themselves contribute to reduced mitochondrial function. Moreover, reduced mitochondrial function can worsen these pathologies as well, resulting in a progressive decline in function. It seems likely the progressive decline in mitochondrial function with time explains why the disease progression is most often dependent on aging. The ability of imaging techniques to track the disease symptoms and biomarkers longitudinally provides the ability to investigate treatments that may intercede in the progression of the disease. A logical place to start is with the agents that preserve mitochondrial function. There are several evident places to attempt this and these include binding of local antioxidants in the mitochondrial membrane, limiting the production of ROS by affecting mitochondrial metabolism such as with the insulin sensitizers, and inhibition of mTOR to relieve the inhibition on autophagy (Fig. 3). Thus far, there are no clinical data to show that any of the approaches will have a meaningful effect in humans. Given the understanding of the progression of the disease and the fact that longitudinal studies are now possible with various kinds of imaging, it is time for a concerted effort to treat mitochondrial dysfunction as soon

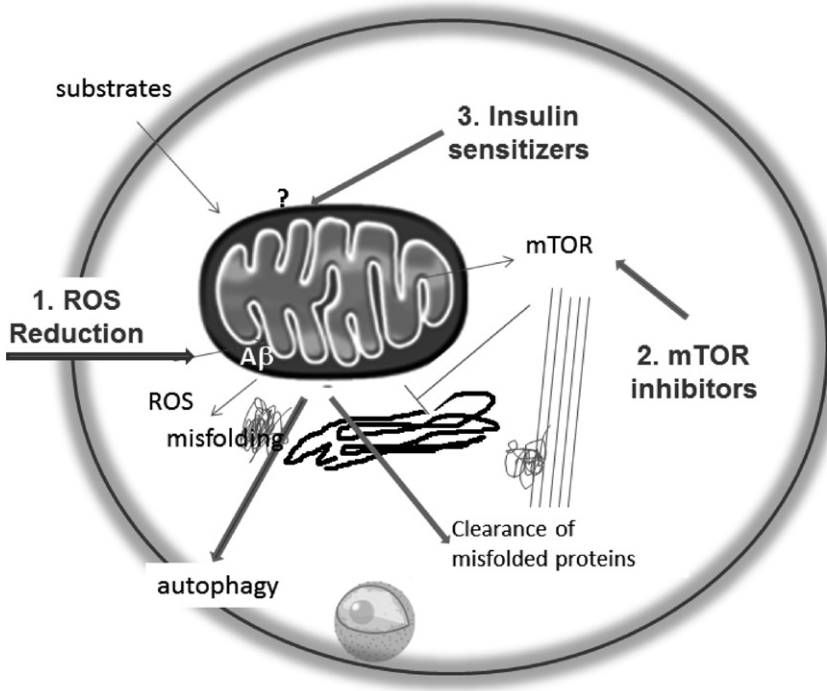

FIGURE 3 **Oxidative metabolism provides increasing amounts of ROS that contribute to the misfolding of proteins and progression of dysfunctional mitochondria.** The ability to clear damaged organelles and proteins is limited by mTOR and loss of mitochondrial functions. Possible routes to intervene in the processes include (1) ways to scavenge or remove ROS; (2) inhibitors of mTOR activity; and (3) reduction of the generation of ROS, which may include the mitochondrial target of insulin sensitizers (mTOT) or other mitochondrial targets that remain to be identified.

as it is detected to determine whether the course of the disease can be changed. There is an obvious need to obtain further insights that allow the identification of more mitochondrial targets, which might provide new, directed drug discovery targets for the treatment of Alzheimer's disease. In our view, the better understanding of the metabolic interface of new insulin sensitizers with mitochondrial function may be a source of such insight. Other approaches may include the delineation of the specific sites of AD pathologies (e.g., amyloid, tau, or lipoproteins) with mitochondrial function.

Conflict of interest: JRC is cofounder and part owner of Metabolic Solutions Development Company, which is currently developing MSDC-0160 for Alzheimer's disease.

Abbreviations

ABAD	amyloid binding alcohol dehydrogenase
ADNI	AD Neuroimaging Initiative
APOE4	Apolipoprotein E-IV
APP	Amyloid Precursor Protein
Aβ	beta amyloid
BACE1	Beta-secretase 1
COX IV	cyclooxygenase IV
Drp1	dynamin-related protein 1
FDG	2-deoxyfluo glucose
GSH	reduced glutathione
IDE	insulin degrading enzyme
PET	positron emission tomography
PreP	presequence protease
ROS	reactive oxygen species

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scyllo-Inositol, Preclinical, and Clinical Data for Alzheimer's Disease

Abstract

Preclinical development of *scyllo*-inositol for the treatment of Alzheimer's disease (AD) has been investigated in both *in vitro* and *in vivo* models with positive results. *scyllo*-Inositol stabilized a small conformer of Aβ42 *in vitro*, neutralized cell derived Aβ trimers and promoted low molecular weight Aβ species *in vivo*. These interactions resulted in decreased neuronal toxicity, increased long-term potentiation (LTP) and ablation of cognitive deficits in multiple mouse models of AD. *scyllo*-Inositol bioavailability, pharmacokinetics, and small animal toxicology studies demonstrated the potential for translation to human patients. The results of Phase I and Phase II clinical trials for AD are presented. Furthermore, the use of this compound for imaging and other amyloid related disorders is discussed.

I. Introduction

scyllo-Inositol has been referred to by a number of names over the course of preclinical and clinical development. Specifically, *scyllo*-inositol has been called *scyllo*-cyclohexanehexol, 1,3,5/2,4,6-cyclohexanehexol, AZD-103, and ELND005, all of which refer to the same compound. The discovery and developmental timeline of *scyllo*-inositol is illustrated in Fig. 1. The use of *scyllo*-inositol as a potential Alzheimer's disease (AD) therapeutic began with the investigations into the mechanism of Aβ-fibril formation, the mechanism of Aβ-mediated toxicity, and the role of Aβ-lipid interactions in these processes (Fenili et al., 2010). Early on it was discovered

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that incubation of Aβ40 and Aβ42 with acidic phospholipids resulted in a random to β-structural transition of Aβ peptides and subsequent disruption of lipid bilayers (McLaurin & Chakrabartty, 1996). Of the acidic phospholipids tested, phosphatidylinositol efficiently induced a β-structure in Aβ42 (McLaurin & Chakrabartty, 1997). To elucidate the component of phosphatidylinositol responsible for β-structural induction, the headgroup,

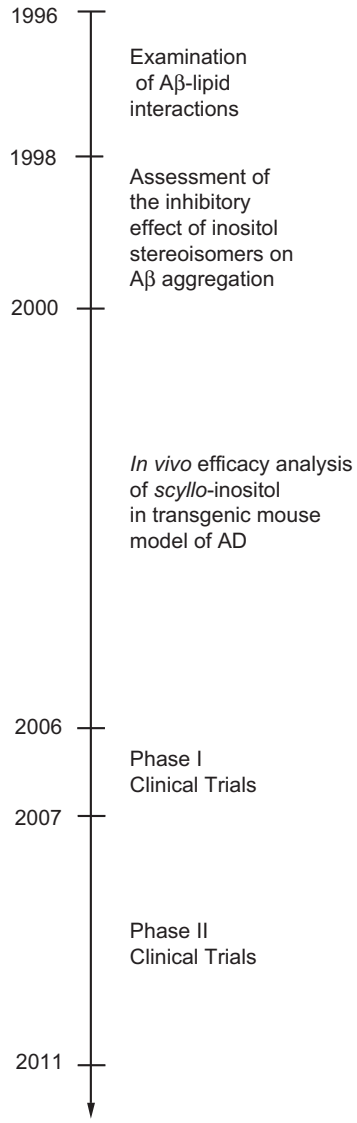

FIGURE 1 Timeline of the discovery and development of *scyllo*-inositol.

fatty acyl chains, and phosphorylation status were examined. It was found that the headgroup of phosphatidylinositol, *myo*-inositol, induced an immediate β-structure transition of Aβ42, but not when phosphorylated (McLaurin et al., 1998). An important finding of this study was that while *myo*-inositol induced a β-structure in Aβ42, it did not lead to the formation of fibrils as was seen when Aβ was incubated alone or with phosphatidylinositol (McLaurin et al., 1998); thus, the beginning of a 14-year journey that still continues.

II. Preclinical Development of *scyllo*-Inositol

The *myo*-inositol-induced formation of stable Aβ42 micelles probed the investigation of other inositol stereoisomers (McLaurin et al., 2000). Inositols are polyols consisting of a six carbon ring structure with a hydroxyl group at each carbon position, also known as cyclohexane-1,2,3,4,5,6-hexol with a chemical formula of $C_6H_{12}O_6$ (Bouveault, 1894; Posternak, 1965). There are nine stereoisomers of inositol based on the orientation of the hydroxyl groups. *myo*-Inositol is the most abundant stereoisomer. Other stereoisomers include *scyllo*-inositol, *cis*-inositol, *epi*-inositol, *allo*-inositol, *muco*-inositol, *neo*-inositol, and the enantiomers *D-chiro*- and *L-chiro*-inositols.

epi-Inositol, *scyllo*-inositol, and *chiro*-inositol were all tested for an effect on Aβ structural transition and prevention of fibril formation (Fenili et al., 2010; McLaurin et al., 2000). Both *epi*-inositol and *scyllo*-inositol, similar to *myo*-inositol, induced a β-structure transition in Aβ42 that did not lead to fibril formation (McLaurin et al., 2000). *chiro*-Inositol on the other hand did not induce a β-structure in Aβ42 and when incubated with Aβ42 lead to the formation of fibrils that were indistinguishable from those formed when Aβ42 was incubated alone (McLaurin et al., 2000). The inositol stereoisomers differ in the orientation of their hydroxyl groups and thus each has a different pattern of hydrogen donors and acceptors (McLaurin et al., 2000). These slight differences in the molecules lead the McLaurin group (2000) to hypothesize that the pattern of hydrogen donors and acceptors may play an important role in determining the structure–activity relationship with Aβ. A more in depth discussion of the structure–function relationship of the inositols is covered later in the chapter.

More recently, two groups confirmed the inhibition of Aβ aggregation by *scyllo*-inositol using very different *in vitro* assays (Park et al., 2011; Zhao et al., 2011). Zhao and colleagues developed novel ELISA assays for screening Aβ aggregation inhibitor compounds based on the fact that Aβ oligomers adopt a conformation that has an exposed N-terminus and buried C-terminus thus providing a measure of differential signal for compounds that affect early stages of oligomer formation (Zhao et al., 2011).

In these assay systems, *scyllo*-inositol was shown to inhibit Aβ42 oligomerization as well as shift the Aβ42 oligomerization equilibrium toward a monomeric state. These results were confirmed using dynamic light scattering. In contrast, Park and colleagues developed a yeast-based screen to identify inhibitors of Aβ42 specific oligomerization (Park et al., 2011). In this yeast model, Aβ42 was fused to the essential functional domain of the translation release factor, Sup35 (MRF), which was overexpressed; this resulted in the formation of SDS-stable low *n*-oligomers. In this system, *scyllo*-inositol decreased oligomer formation by greater than 50% and rescued the growth defect without an increase in cell death. These results are consistent with the previous *in vitro* studies that demonstrated *scyllo*-inositol induced inhibition of Aβ oligomerization (McLaurin et al., 2000; Townsend et al., 2006).

Since the accumulation of Aβ oligomers/fibrils is believed to be a key component in AD pathology (Karran et al., 2011), the effect of compounds on the inhibition of fibrillogenesis is of great interest. In order for any compound to undergo further investigation as a potential therapeutic, the toxicity of the complex formed in the presence of Aβ must be tested (Shaw et al., 2011). It is well documented that Aβ oligomers are toxic, therefore compounds that favor the stabilization of oligomers may enhance toxicity as was seen for the naphthalene sulfonates and some *N*-methylated peptides (Ferrao-Gonzales et al., 2005; Kokkoni et al., 2006); however, off-fiber pathway oligomers are not toxic as was shown with resveratrol and RS-0406 compounds (Feng et al., 2009; Walsh et al., 2002). The differentiation of these two oligomers by structure alone is not always possible and hence the added information given by the toxicity assay is necessary for clinical development (Shaw et al., 2011). Preincubation of Aβ42 with *myo*-inositol, *epi*-inositol, or *scyllo*-inositol led to the increased survival of nerve growth factor (NGF)-differentiated PC-12 cells and primary neuronal cultures compared to neurons exposed to Aβ42 alone (McLaurin et al., 2000). Further, when Aβ42 was incubated with PC-12 cells, it accumulated on the surface of the cells. However, *myo*-, *epi*-, and *scyllo*-inositol inhibited the accumulation of Aβ42 on the cell surface (McLaurin et al., 2000). This led to the proposal that the observed reduction of toxicity may be in part a result of the decreased interaction of Aβ with the cell membrane (McLaurin et al., 2000). These combined results demonstrated that three of the nine inositol stereoisomers demonstrated *in vitro* properties that are conducive to further investigation.

Since *myo*-, *epi*-, and *scyllo*-inositol were all successful at inhibiting fibrillogenesis *in vitro*, their efficacy *in vivo* was assessed. These three inositol stereoisomers were administered to a transgenic mouse model of AD—the TgCRND8 model (Fenili et al., 2010; McLaurin et al., 2006). The TgCRND8 mouse model is considered an aggressive model due to the overexpression of the human amyloid precursor protein (APP695) that contains

the "Swedish" mutation (K670N, M671L) and the "Indiana" mutation (V717F) (Chishti et al., 2001). These mice express a high Aβ42:40 ratio that results in the development of amyloid deposits and cognitive deficits by 3 months of age (Chishti et al., 2001). Further, these mice have accelerated mortality, a common consequence of AD in human patients (Chishti et al., 2001; McLaurin et al., 2006). Treatment of TgCRND8 mice with *myo*-inositol was not effective since no significant cognitive benefit was observed, which may not be surprising because *myo*-inositol levels are highly regulated within the central nervous system (CNS) (McLaurin et al., 2006; Fenili et al., 2007). Treatment with *epi*-inositol appeared to have effects at the early stages of disease, 4 months of age; however, as the disease progressed no beneficial effects were detected (McLaurin et al., 2006). In contrast to *myo*- and *epi*-inositol, *scyllo*-inositol treatment improved AD-like pathology when given prophylactically starting at 6 weeks of age and continuing until 4 and 6 months of age (McLaurin et al., 2006). Treatment of TgCRND8 mice with *scyllo*-inositol increased survival from 42% to 72% at 6 months of age ($p = 0.02$) and the treated mice showed a complete improvement of cognitive deficits when assessed by the Morris water maze test of spatial memory at both ages (McLaurin et al., 2006).

Further confirmation of improved cognition in the treated TgCRND8 mice was the reduction of synaptic toxicity illustrated by 146% increase in synaptophysin positive boutons and cell bodies in the hippocampus at 6 months of age (Fenili et al., 2010; McLaurin et al., 2006). *scyllo*-Inositol treatment reduced total Aβ40 ($p < 0.001$) and Aβ42 ($p < 0.05$) and decreased parenchymal plaque load throughout the brain ($p < 0.05$) (McLaurin et al., 2006). Fig. 2 shows Aβ plaques, in brown, at 6 months of age in TgCRND8 untreated (A and B) and *scyllo*-inositol treated (D and E) mice in the hippocampus and cortex, respectively. High magnification images of untreated (2C) and *scyllo*-inositol treated (2F) TgCRND8 brain illustrate the decrease in mean plaque size as a result of *scyllo*-inositol treatment. Treatment also decreased the size of cerebrovascular Aβ deposits and reduced the percentage of brain area covered by vascular amyloid. Treatment with *scyllo*-inositol reduced the amount of high molecular weight Aβ species and increased the levels of trimeric and monomeric Aβ species, suggesting that the beneficial effects of *scyllo*-inositol are due to the inhibition and/or disaggregation of high molecular weight Aβ species (McLaurin et al., 2006). Lastly, *scyllo*-inositol improved the neuroinflammatory status of treated TgCRND8 mice through the reduction of microgliosis and astrogliosis (McLaurin et al., 2006). Decreased astrogliosis is shown in Fig. 2 where *scyllo*-inositol treatment decreased the number of reactive astrocytes (labeled in red) throughout the hippocampus (2A) and cortex (2B) compared to that of the controls (2D, 2E). As shown in high magnification, astrogliosis is reduced surrounding Aβ plaques as well as nonplaque associated regions (2C, 2F).

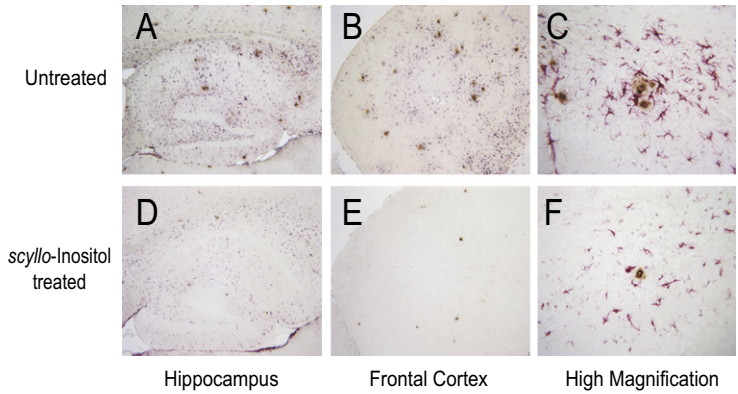

FIGURE 2 ***scyllo*-Inositol treatment decreased Aβ plaque load and reduced astrogliosis in the TgCRND8 brain at 6 months of age.** Aβ plaques are brown and reactive astrocytes are red. Aβ plaques and astrocytes in the hippocampus and cortex of untreated TgCRND8 brain are shown in A and B respectively. Decreased Aβ plaque load and astrogliosis as a result of *scyllo*-inositol treatment in the hippocampus and cortex are shown in D and E. C and F show the differences between control and *scyllo*-inositol treatment in high magnification. (Modified from McLaurin et al., 2006). For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

It is evident that *scyllo*-inositol can inhibit the development of AD-like pathology in TgCRND8 mice when administered prior to the expression of the AD-like phenotype. However, for *scyllo*-inositol to be a treatment for AD, it would need to be effective once the disease has already begun, since AD is initiated approximately 10 years prior to the onset of clinical symptoms (Shim & Morris, 2011). To determine therapeutic potential, *scyllo*-inositol was administered to TgCRND8 mice for 28 days starting at 5 months of age (Fenili et al., 2010; McLaurin et al., 2006). At this age, the mice have significant Aβ and plaque loads as well as cognitive deficits (Chishti et al., 2001). Assessment of these mice at 6 months of age, using the Morris water maze test, showed that treated TgCRND8 mice had significantly improved performance compared to untreated TgCRND8 mice ($p = 0.01$), and their performance was not significantly different from non-transgenic littermates ($p = 0.11$) (McLaurin et al., 2006). Similar to the results of the prophylactic experiments, *scyllo*-inositol treatment after disease onset reduced insoluble Aβ40 ($p < 0.05$) and Aβ42 ($p < 0.05$) levels in the brain and significantly reduced plaque burden ($p < 0.05$) (McLaurin et al., 2006). Overall the beneficial effects were similar to those from the prophylactic studies (McLaurin et al., 2006).

Following the publication of the effects of *scyllo*-inositol on the AD-like phenotype in TgCRND8 mice, further investigation utilizing different *in vitro* and *in vivo* models confirmed the effects of *scyllo*-inositol. Townsend and colleagues found that *scyllo*-inositol could prevent Aβ-oligomer-induced inhibition of long-term potentiation (LTP) in hippocampal mouse brain

slices (Townsend et al., 2006). When *scyllo*-inositol was preincubated with Aβ or applied to cells producing Aβ, inhibition of LTP normally caused by Aβ was significantly reduced (Townsend et al., 2006). However, when *scyllo*-inositol was applied to the brain section after Aβ was applied, there was no protection of LTP (Townsend et al., 2006). The authors confirmed that protection of LTP by *scyllo*-inositol was a result of neutralization of secreted Aβ trimers (Townsend et al., 2006). Further support for *scyllo*-inositol-induced neutralization of Aβ oligomers comes from the observation that simultaneous application of *scyllo*-inositol and Aβ oligomers prevented oligomer-induced decrease in dendritic spine density (Shankar et al., 2007). These *in vitro* studies correlate well with the rescue of cognitive deficits in the TgCRND8 mouse studies.

The effect of *scyllo*-inositol to reverse or diminish Aβ-induced cognitive deficits in an acute model of Aβ-toxicity was examined using *in vivo* studies in rats (Townsend et al., 2006). Intracerebroventricular (ICV) injection of Aβ in rats increases switching and perseveration errors in the alternating lever cyclic ratio assay, which is a test of complex reference memory (Townsend et al., 2006). When *scyllo*-inositol is incubated with Aβ prior to ICV injection both types of errors are decreased. These errors also returned to baseline when *scyllo*-inositol was orally administered for 3 days prior to ICV injection of Aβ (Townsend et al., 2006). These findings suggest that *scyllo*-inositol is effective in an acute AD model.

It is evident from imaging studies that the amyloid load and presumably the Aβ load in patients varies greatly (Devanand et al., 2010). Since we are presently unable to predict the Aβ load in patients with AD, understanding the effectiveness of a potential compound in various mouse models of different genetic backgrounds and varying Aβ loads is important for translation to a highly variable AD population. In order to determine the effect of *scyllo*-inositol in a more aggressive transgenic model of AD, the treatment of disease-bearing PS1 (M146L + L286V) × TgCRND8 mouse (PS1 × APP) (Chishti et al., 2001) with *scyllo*-inositol for 4 weeks was examined (DaSilva et al., 2009; McLaurin, unpublished results). The PS1 × APP mouse model exhibits high Aβ load and significant plaque burden by 1 month of age, and continues to rapidly accumulate Aβ to that of end-stage AD patients by 2 months of age; this is in comparison to TgCRND8 singly-transgenic mice which have Aβ load equivalent to end-stage AD patients at 7 months of age (Chishti et al., 2001). The high expression of Aβ is the result of incorporation of two familial AD mutations into both APP and presenilin-1. The rapid accumulation of Aβ plaques is the result of the high Aβ42:Aβ40 ratio, 14:1, and the greater propensity of Aβ42 to aggregate. Since Aβ is produced at supra-physiological levels, any significant changes in amyloid load seen in this model would be indicative of a potent compound.

Effective inhibition of Aβ plaque deposition once treatment was initiated demonstrated that even at supra-pathological Aβ levels, *scyllo*-inositol

was highly potent (DaSilva et al., 2009). Both plaque count and percentage brain area occupied by plaques were reduced by 50% (Fig. 3A and B), which is greater than the reductions seen in TgCRND8 mice treated at 5 months of age for 28 days (McLaurin et al., 2006). Insoluble Aβ40 and Aβ42 and soluble Aβ42 were also significantly reduced after *scyllo*-inositol treatment with reductions that were equivalent to those observed in TgCRND8 mice (McLaurin et al., 2006). Previous studies demonstrated that *scyllo*-inositol decreased high molecular weight Aβ oligomers and populated trimeric and monomeric Aβ species thereby rescuing cognitive deficits in this model (McLaurin et al., 2006). Here soluble oligomeric species (greater than 40kDa) increased following treatment, while monomeric, dimeric, and trimeric Aβ aggregates were also increased (Fig. 3C). The high level of Aβ expression in this mouse model may preclude effective removal of soluble Aβ species. Moreover, these results correlate with previous vaccine studies in TgAPP mice, which demonstrate a robust decrease in Aβ plaques and cognitive improvements with no change in total brain Aβ levels (Janus et al., 2000).

Recent reports have pointed to soluble Aβ oligomers as the neurotoxic species capable of inhibiting LTP, learning, and memory (Klyubin et al., 2005; Townsend et al., 2006; Walsh et al., 2002). Ultrastructurally soluble Aβ oligomers have been localized to cell processes in AD brains (Kokubo et al., 2005), and appear to be targeted to synapses in cultured hippocampal neurons (Lacor et al., 2004). Exposure of neurons to oligomers causes abnormal spine morphology, decreased spine density, and decreased expression of synaptic markers such as synaptophysin (Ishibashi et al., 2006; Lacor et al., 2007; Shankar et al., 2007). Indeed, decreases in synaptophysin, syntaxin, and dynamin-1 have been correlated with cognitive decline in transgenic models of AD (Kelly et al., 2005; Oakley et al., 2006). Treatment with *scyllo*-inositol after the appearance of Aβ oligomers (at 1 month) appears to restore synaptic architecture, as seen by increases in the expression of the presynaptic markers synaptophysin, syntaxin, synapsin, and dynamin-1 (Fig. 3D; DaSilva et al., 2009; McLaurin unpublished results). This was seen irrespective of the increased levels of monomers, dimers, trimers, and higher molecular-weight oligomers (>40kDa). This is in agreement with previous findings where treatment with *scyllo*-inositol increased synaptophysin levels in both prophylactic and treatment paradigms (McLaurin et al., 2006). Thus, *scyllo*-inositol protected against synaptic dysfunction in PS1 × APP mice and demonstrated the potency of *scyllo*-inositol in an aggressive model of AD.

Another mouse model, 5 × FAD, which contains five familial AD mutations—APP 695 K670N/M679L (Swedish), I716V (Florida), V717I (London), and PS1 M146L and L286V mutations, was utilized as an alternate model of Aβ pathology (Oakley et al., 2006). These mice show cerebral amyloid plaques and gliosis by 2 months of age and have increased Aβ42

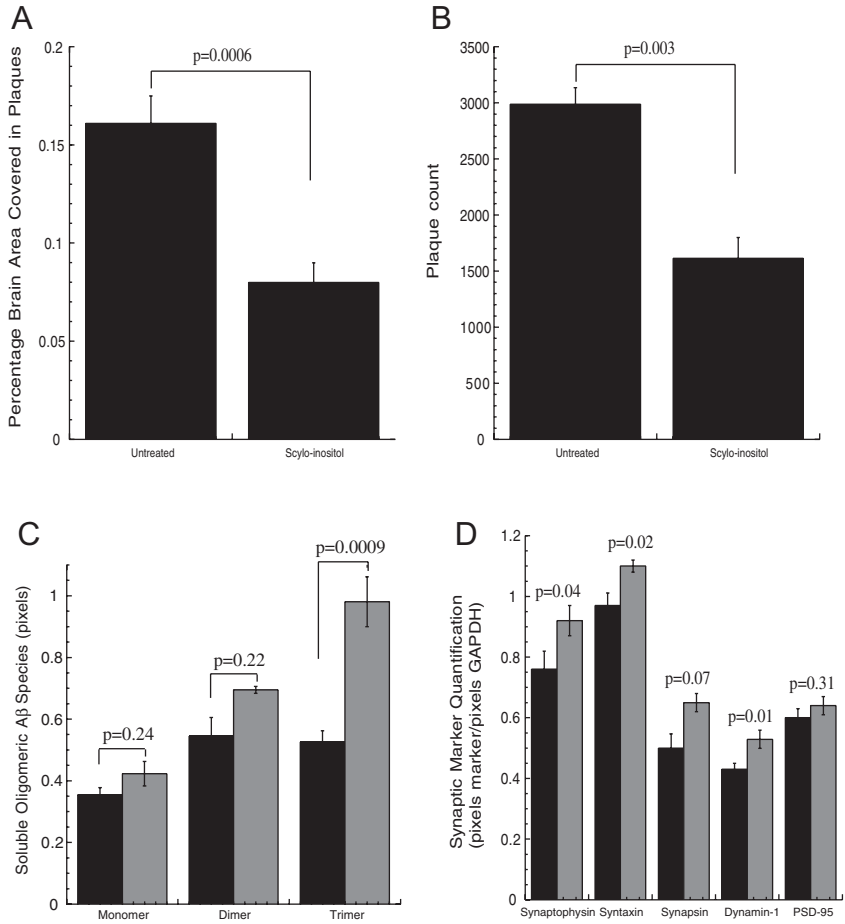

FIGURE 3 **Evaluation of the effect of *scyllo*-inositol on plaque load and synaptic health in an aggressive transgenic mouse model of AD.** *scyllo*-Inositol treatment for 4 weeks starting at 4 weeks of age in PS1 × APP transgenic mouse model of AD decreased percentage brain area covered by plaques (A) and plaque count (B) by approximately 50%. *scyllo*-Inositol also increased monomeric, dimeric and trimeric Aβ (D) as well as rescued synaptic architecture as shown by the increase in synaptic markers synaptophysin, syntaxin, synapsin and dynamin-1 (E).

levels detected as early as 1.5 months of age. The 5 × FAD mice first develop plaques in the cortex and subiculum, which then progress to the hippocampus, thalamus, brain stem, and olfactory bulbs. Further, these mice display neuronal loss and exhibit memory deficits between 4 and 5 months of age (Oakley et al., 2006). Aytan and colleagues administered *scyllo*-inositol to 5 × FAD mice for 1 month after amyloid pathology was evident (Aytan et al., 2011). The *scyllo*-inositol treated mice were compared to untreated 5 × FAD wild-type mice and 5 × FAD treated mice in combination with

scyllo-inositol and R-flurbiprofen. Image analyses demonstrated that *scyllo*-inositol treated mice had 38% and 34% reduction in Aβ42 and Aβ40 containing deposits as well as parallel peptide reductions as measured by ELISA. These reductions were accompanied by improvement in performance in the radial arm water maze task of spatial memory. Surprisingly, treatment with *scyllo*-inositol in the presence of R-flurbiprofen was less effective both cognitively and pathologically than *scyllo*-inositol treatment alone (Aytan et al., 2011). These results further demonstrate the effectiveness of *scyllo*-inositol in mouse models containing varying familial mutations, genetic backgrounds, and Aβ burdens.

III. Sources of *scyllo*-Inositol

Alzheimer's Disease International estimated in 2009 that there were 36 million people suffering from dementia worldwide and this number will increase to 66 million by 2030 and 115 million by 2050. In 2010 alone, worldwide cost for dementia was $604 billion (Prince et al., 2011). For a naturally occurring compound to be used as a pharmaceutical to treat AD, it needs to be available in large quantities. Furthermore, the isolation procedures or synthetic pathways necessary to achieve this requirement must also be done in a reasonable time frame and at reasonable cost.

A. Natural Sources

scyllo-Inositol was first discovered in sharks and skates in 1858 by Staedler and Frerichs; it was extracted from the kidney, liver, spleen, and gills of the shark *Scyllium canicula* and skates *Raja batis* and *Raja clavata* (Fenili et al., 2010; Staedler & Frerichs, 1858). *scyllo*-Inositol is present in all organs of the skate *Raja erinacea*, with highest levels in the liver and kidney; it is found at a much higher concentration in most organs of skates compared to *myo*-inositol, except the brain and the peripheral nerve (Sherman et al., 1978). Skates have slow growth and low reproductive rates making them a poor choice for *scyllo*-inositol isolation in bulk quantities (Mcphie & Campana 2009; Williams et al., 2011). *scyllo*-Inositol is also found in a variety of plant tissues including coconut, soursop, flowers of dogwood, and the bark of white and English oak (Goodson, 1920; Hann & Sando, 1926; Muller, 1907; Muller, 1912). Alternatively, *scyllo*-inositol has been reported in grapes, some citrus fruit, and vegetables of the Apiaceae family (Fenili et al., 2010; Sanz et al., 2004; Soria et al., 2009). It is even found in insects such as locusts, cockroaches, and blow flies (Candy, 1967). Although many higher plants express compounds in quantities that are sufficient for raw materials for scientific and commercial applications, there are exceptions such as inositol and β-carotene which are very expensive due to the

extraction, isolation, and purification process (Balandrin et al., 1985; Fenili et al., 2010).

B. Chemical Synthesis

Besides natural sources, *scyllo*-inositol can be synthesized chemically. *scyllo*-Inositol has been synthesized historically by a number of synthetic routes including from halobenzenes, tetrahydroxyquinone, sugars, and other inositols (Anderson & Wallis, 1948; Angyal et al., 1995; Kowarski & Sarel, 1973; Mandel & Hudlicky 1993; Watanabe et al., 1987; Tagliaferri et al., 1990). However, these methodologies have low efficiencies for industrial scale use. More recent methods demonstrate increased efficiencies and are discussed here. One methodology involves the didehydroxylation of *myo*-inositol via the intermediate conduritol B to produce gram scale quantities of *scyllo*-inositol. In this method, *myo*-inositol diols are converted to conduritol B, which was epoxidized, followed by ring opening to produce *chiro*- and *scyllo*-inositol isomers with the *scyllo*-inositol isomer in much lower yield (Chung & Kwon, 1999). Although this synthetic route produced gram scale quantities, the yield of *scyllo*-inositol is only 16%.

To increase yield, Podeshwa and colleagues employed the production of inositol stereoisomers from enantiomerically pure building blocks (Podeshwa et al., 2003). These building blocks were diacetoxy-dibromocyclohex-5-ene (+) or (−), which are easily made from *p*-benzoquinone. Similar to the method by Chung and Kwon, diacetoxy-dibromocyclohex-5-ene (+) is converted to a conduritol B intermediate, which undergoes epoxidation. Regioselective opening of the epoxide yields a *scyllo*-isomer, which becomes *scyllo*-inositol after hydrogenation (Podeshwa et al., 2003). This method produces *scyllo*-inositol in much higher yield, yet requires purification steps.

To improve the yield of *scyllo*-inositol, methodological development was further employed. The use of 6-deoxyhex-5-enopyranosides as the starting compound instead of *myo*-inositol (Takahashi et al., 2001), combined with Ferrier-II carbocyclization, efficiently produces chiral-substituted cyclohexanones. This reaction was catalyzed by palladium dichloride to produce β-hydroxycyclohexanones. Stereoselective reduction of β-hydroxycyclohexanones with $NaBH_4$ resulted in *scyllo*-inositol in good yield, 86% of starting material (Takahashi et al., 2001). This method has the advantage of abundant starting material at low cost and a controlled efficient production of *scyllo*-inositol.

To further improve the yield of *scyllo*-inositol, Sarmah and Shashidhar developed another synthetic scheme (Sarmah & Shashidhar, 2003). The overall yield to produce *scyllo*-inositol from *myo*-inositol via an orthoformate intermediate was 64% using this method. In this method, *myo*-inositol was

first converted to *myo*-inositol orthoformate using a convenient high-yielding methodology without the use of time consuming chromatography (Praveen & Shashidhar, 2001). *myo*-Inositol orthoformate was then benzoylated to yield 2-benzoate. Sulfonylation of 2-benzoate with subsequent Swern oxidation and reduction produced *scyllo*-ditosylate. Through a series of methanolysis, acetylation, and aminolysis, *scyllo*-inositol orthoformate was made. The final step of hydrolysis with aqueous acid converted *scyllo*-inositol orthoformate to *scyllo*-inositol (Sarmah & Shashidhar, 2003). This methodology improved upon the synthetic strategies based on *myo*-inositol, but it still had a lower yield than some of the previous synthetic pathways.

Although chemical synthesis of pure *scyllo*-inositol has improved with yields up to 86% from starting material, this represents a labor intensive process including purification steps.

C. Biological Synthesis

Biological synthesis of *scyllo*-inositol started with the discovery of enzymes that convert the abundant *myo*-inositol to the other stereoisomers (Larner et al., 1956; Ramaley et al., 1979). In 1977, Hipps and colleagues discovered an epimerase from bovine brain extract, specifically in the unbound DEAE-cellulose fraction, which converts *myo*-inositol to *neo*- and *scyllo*-inositol (Hipps et al., 1977). This epimerase functions at an optimal pH of 9.5 in the presence of dithiothreitol. Incubation of the epimerase with NADP+ allows for a much greater conversion of *myo*-inositol to *neo*- and *scyllo*-inositol than with NAD+ (Hipps et al., 1977). Although the presence of the epimerase in bovine brain has been known for a long time, utilization of this enzyme for translation into biological or industrial use has not been reported.

Evolutionary studies have shown that archaea, several bacteria, and eukaryotes synthesize and utilize *myo*-inositol for various functions (Michell, 2008). More recently it has also been recognized that *scyllo*-inositol can be utilized as readily as *myo*-inositol in these organisms (Michell, 2008). Gram positive bacteria, *bacillus subtilis*, have a unique inositol metabolism pathway that involves *myo*-, *chiro*-, and *scyllo*-inositols (Holub, 1986; Yoshida et al., 2008). This bacterial strain was genetically modified in 2006 to generate a cell factory for the production of *chiro*-inositol after the discovery that *chiro*-inositol may have a benefit for diabetes (Yoshida et al., 2006). More recently, this same system, *bacillus subtilis,* was modified as a cell factory to convert *myo*-inositol to *scyllo*-inositol as an inexpensive method to produce *scyllo*-inositol (Yamaoka et al., 2011). Under normal conditions, *bacillus subtilis* convert *myo*-Inositol to *scyllo*-inosose by the *myo*-inositol dehydrogenase, IolG, coupled with the reduction of NAD^+ to NADH. Multiple step degradation of *scyllo*-inosose produces the final *myo*-inositol degradation products—dihydroxyacetone

phosphate and acetyl-CoA. Alternatively, *scyllo*-Inosose can be converted to *scyllo*-inositol by the distinct *myo*-inositol dehydrogenase, IolW, coupled with NADPH oxidation to $NADP^+$. The identification of the *iolE41* missense mutation allele in genetically modified *Bacillis subtilis* results in interference with the degradation of *scyllo*-inosose. The resultant intracellular accumulation of *scyllo*-inosose increased the production of *scyllo*-inositol from *scyllo*-inosose by IolW (Yamaoka et al., 2011). This cell factory method of *scyllo*-inositol production converts close to 10g/L of *myo*-inositol to *scyllo*-inositol in 48h.

An alternate method for *scyllo*-inositol production involves the bioconversion of *myo*-inositol to *scyllo*-inositol using microorganisms optimized for industrial-scale production (Reddy et al., 2011). In this bioconversion, microorganisms of the *Acetobactor* and *Burkholderia* genera were used to convert *myo*-inositol to *scyllo*-inosose and *scyllo*-inositol in a fermentation mixture. This mixture is then treated with a base and subjected to heat to degrade *scyllo*-inosose and lyse the cells. The remaining bioconversion product, *scyllo*-inositol, is reacted with boric acid and sodium hydroxide to form *scyllo*-inositol-diborate-disodium salt complex. This complex is then hydrolyzed to yield crude *scyllo*-inositol before crystallization to produce the final product of *scyllo*-inositol.

Yamaguchi and colleagues identified a novel NAD^+-independent *myo*-inositol 2-dehydrogenase in *Acetobacer*, strain AB10253, which catalyzes an efficient conversion of *myo*-inositol to *scyllo*-inosose (Yamaguchi et al., 2004). AB10253 or any microorganisms containing NAD^+-independent *myo*-inositol 2-dehydrogenase can be used to produce *scyllo*-inositol from *myo*-inositol. The resulting product from the bioconversion of *myo*-inositol, *scyllo*-inosose can be reduced to *scyllo*-inositol via *scyllo*-inositol dehydrogenase in a NADH/NADPH-dependent manner. Once *scyllo*-inositol is produced in the microorganism, it is precipitated to form a complex of low solubility. This complex is then dissolved in acid and purified by either ion exchange resin or a water-soluble organic solvent extraction to produce *scyllo*-inositol.

These combined bioconversion studies show the potential for the production of pharmaceutical quantities of *scyllo*-inositol at reasonable costs. The method by Yamuguchi and colleagues was utilized for both the preclinical development and Phase I and II clinical trials of *scyllo*-inositol.

IV. Bioavailability and Metabolism

The next major hurdle normally encountered for the development of CNS drugs is brain bioavailability. In fact many active compounds fail to proceed further in development due to the lack of adequate CNS bioavailability. For an aging population and long-term delivery, oral bioavailability

is the most convenient and readily compliant method for drug administration. In the preclinical studies, oral administration of *scyllo*-inositol had beneficial effects; however, the mechanism of transport of *scyllo*-inositol across the blood–brain barrier (BBB) was unknown.

To prove that orally administered *scyllo*-inositol was acting within the CNS, gas chromatography/mass spectrometry was performed to determine *scyllo*-inositol levels in the CSF and brain after oral administration in the TgCRND8 mouse model (Fenili et al., 2007). Analysis of *scyllo*-inositol *ad libitum* treatment showed a 16-fold increase ($p < 0.001$) in CSF *scyllo*-inositol levels and a 7.6-fold increase ($p < 0.001$) in brain *scyllo*-inositol levels (Fenili et al., 2007). *scyllo*-Inositol treatment did not significantly alter *myo*-inositol levels in the CSF, which is advantageous since *myo*-inositol is an organic osmolyte and is involved in cell signaling in the brain (Fenili et al., 2007). Furthermore, *scyllo*-inositol levels were significantly higher after *ad libitum* treatment compared to a single daily dose. This would suggest that receiving multiple doses throughout the day over the course of treatment may allow for high CNS *scyllo*-inositol levels to be maintained. A twice daily gavage regiment resulted in similar cognitive and pathological readout measures as was seen for *ad libitum* administration (McLaurin et al., 2006).

scyllo-Inositol levels in the brain can also be measured using magnetic resonance spectroscopy (MRS). MRS is useful for detecting neurochemical changes in the brain of AD mouse models and has been utilized to distinguish AD from other dementias in patients (Watanabe et al., 2010). Using two mouse models of AD, *scyllo*-inositol levels were measured from brain tissue extracts and intact hippocampal and cortical tissue (Choi et al., 2010). The mouse models utilized in this study were the Tg2576xPS1[M146V] (Holcomb et al., 1998) while the second model was a triple transgenic, 3XTg, that expresses human APP [Swedish mutation], PS1 [M146V] and Tau [P301L] mutations (Oddo et al., 2003). The models differ not only in the inclusion of the Tau P301L mutation but also in the expression level of Aβ and genetic background. *scyllo*-Inositol was administered *ad libitum* for a final dose of 3.3mg/Kg/day, as was previously shown to be effective in mice (McLaurin et al., 2006). Isolation of tissue homogenates for solution MRS and intact brain regions for high resolution magic angle spinning spectroscopy analyses of treated and untreated mice were harvested (Choi et al., 2010). Analyses of tissue homogenates demonstrated a 3-fold increase in *scyllo*-inositol treated versus untreated mice, which was confirmed using magic angle spectroscopy. *scyllo*-Inositol levels were increased in both the hippocampus and the frontal cortex with higher levels found in the hippocampus in both mouse models (Choi et al., 2010). These studies confirmed the increase in *scyllo*-inositol in TgCRND8 mice.

The oral availability of *scyllo*-inositol was further tested in long-term toxicology studies in rats and dogs, which demonstrated that *scyllo*-inositol

was completely orally bioavailable (http://www.transitiontherapeutics.com/media/news.php). Further development required understanding the pharmacokinetic properties of *scyllo*-inositol in the brain (Quinn et al., 2009). In order to determine brain and CSF exposure, Spraque-Dawley rats were gavaged with *scyllo*-inositol at three doses twice daily for 5 days. The concentration of *scyllo*-inositol in plasma, CSF, and frontal cortices was determined at 1–24 h after the last dose by gas-chromatography mass spectrometry (Quinn et al., 2009). Plasma *scyllo*-inositol levels increased disproportionately to dose as did CSF and brain levels. The CSF *scyllo*-inositol concentration was half plasma levels while brain was 10-fold increased over plasma and remained constant over the course of the experiment. The CSF levels decayed in parallel with the plasma levels. The absorption of *scyllo*-inositol was rapid with time of maximum uptake within 1–3 h of dosing. Brain concentrations reached 4-, 8-, and 12-fold increased levels over endogenous *scyllo*-inositol when dosed at 5, 15, and 30mg/Kg BID (Quinn et al., 2009). The combined rodent studies show that *scyllo*-inositol is CNS bioavailable, which would suggest that *scyllo*-inositol is either actively transported or passively diffused into the brain.

In 1976, it was recognized that transport of *myo*-inositol into the CNS was through a saturable transport system in the choroid plexus (Spector, 1976). Subsequently, the three inositol transporters were reported: sodium/*myo*-inositol transporter 1 (SMIT1), sodium/*myo*-inositol transporter 2 (SMIT2), and proton/*myo*-inositol transporter (HMIT). SMIT1 was the first of the inositol transporters to be discovered (Kwon et al., 1992). The discovery of SMIT2 and HMIT, similar to SMIT1, occurred through further investigations into cellular osmoregulation and general inositol transport (Kwon et al., 1992; Coady et al., 2002; Uldry et al., 2001). More recently the role of SMIT1/2 and HMIT in *scyllo*-inositol CNS bioavailablity and how this may impact the potential therapeutic effects of *scyllo*-inositol for AD have been investigated (Fenili et al., 2011).

A. Sodium/*myo*-Inositol Transporter 1

The SMIT1 is member number three of the solute carrier family five, thus is also referred to as SLC5A3 (Berry et al., 1995). SMIT1 has greater than 93% homology across human, mouse, canine, and bovine species (McVeigh et al., 2000). SMIT1 mRNA has been found in human brain, kidney, placenta, pancreas, and lung tissue, as well as in heart and skeletal muscle (Berry et al., 1995; Fenili et al., 2010). Analysis of the rat brain revealed that the choroid plexus contained the highest levels of SMIT1 mRNA, with expression also in the pineal gland, area postrema, hippocampus, locus coeruleus, suprachiasmatic nucleus, olfactory bulb, and the purkinji and granular cell layers of the cerebellum (Inoue et al., 1996). Further,

SMIT1 expression was present in neurons and glia-like cells across the rat brain (Inoue et al., 1996). The majority of *myo*-inositol transport into the brain likely occurs through SMIT1, as SMIT1–/–- mouse pups show a 92% reduction in brain *myo*-inositol and do not survive for long after birth (Berry et al., 2003). In addition, SMIT1 +/– mice show a 15% reduction of *myo*-inositol in the cortex and a 25% reduction in the hippocampus (Shaldubina et al., 2007) suggesting that SMIT1 is also important in transport of *myo*-inositol to these areas of the brain.

SMIT1 cotransports Na^+ and *myo*-inositol in a ratio of 2:1 (Matskevitch et al., 1998), but as shown in *Xenopus* oocytes expressing SMIT1, this transporter has the ability to transport numerous other sugars with the following specificity; *myo*-inositol = *scyllo*-inositol > L-fucose > L-xylose > L-glucose = D-glucose = alpha-methyl-D-glucopyranoside > D-galactose = D-fucose = 3-O-methyl-D-glucose = 2-deoxy-D-glucose > D-xylose (Hager et al., 1995; Fenili et al., 2010). The equal affinity of SMIT1 for *myo*-inositol and *scyllo*-inositol is unique among the inositol transporters (Fenili et al., 2010). SMIT1 is expressed on the plasma membrane, and in polarized cells it allows for *myo*-inositol to be taken up on the basolateral side of the cell (Kwon et al., 1992). In porcine choroid plexus cells *myo*-inositol was transported from the basolateral to the apical side of cells, resembling transport that may occur from blood to cerebral spinal fluid (Hakvoort et al., 1998).

B. Sodium/*myo*-Inositol Transporter 2

The most recently discovered inositol transporter is SMIT2, also known as member 11 of the solute carrier family five (Lin et al., 2009). The highest expression of SMIT2 mRNA has been found in heart, skeletal muscle, kidney, liver, and placenta tissue, with weaker expression also reported in the brain of humans (Roll et al., 2002). The SMIT2 sequence is 43% similar to that of SMIT1 and similarly cotransports Na^+ and *myo*-inositol in a 2:1 ratio (Coady et al., 2002; Fenili et al., 2010). However, these proteins show some interesting differences in their transport properties. SMIT2 exhibits stereospecificity transport of sugars, transporting D-glucose and D-xylose, but not their L-enantiomers (Coady et al., 2002; Ostlund et al., 1996). This is unlike SMIT1, which shows no glucose stereospecificity (Coady et al., 2002; Ostlund et al., 1996). Further, SMIT2 shows similar affinity for *myo*-inositol and D-*chiro*-inositol (Lin et al., 2009), while SMIT1 does not appear to transport D-*chiro*-inositol (Coady et al., 2002; Ostlund et al., 1996).

Both SMIT1 and 2 are expressed on the plasma membrane; however, in polarized cells such as Madin-Darby canine kidney cells, SMIT2 is located on the apical membrane while SMIT1 is located on the basolateral membrane (Bissonnette et al., 2004; Fenili et al., 2010). In these same cells,

hyperosmotic conditions increase both SMIT1 and 2 activities. Interestingly, SMIT1 activity is increased at low hyperosmotic levels and SMIT2 activity is increased at higher hyperosmotic levels (Bissonnette et al., 2008). In addition, SMIT2 activity appears to be induced quicker and peaks faster than SMIT1 (Bissonnette et al., 2008). These findings suggest that SMIT1 and SMIT2 may work together to regulate inositol levels and osmolarity within tissues.

C. Proton/*myo*-Inositol Transporter

The HMIT is one of the 13 members of the facilitative glucose transporter family of proteins (Fenili et al., 2010; Uldry et al., 2001; Zhao & Keating, 2007). Despite belonging to a family of glucose transporters, HMIT does not transport glucose or any of the related hexoses (Uldry et al., 2001). HMIT is a proton/*myo*-inositol symporter; however, rat HMITs have been shown to also transport *scyllo*-inositol, *muco*-inositol, and *chiro*-inositol, but to a lesser extent than *myo*-inositol (Uldry et al., 2001). The 90% homology of rat and human HMIT would suggest similar transport properties between these species (Fenili et al., 2010).

HMIT is expressed in small amounts in adipose tissue and the kidney, but its expression is greatest in the brain (Uldry et al., 2001). The highest expression of HMIT has been found in the cerebral cortex, hippocampus, hypothalamus, cerebellum, and brainstem (Uldry et al., 2001). HMIT expression in the brain has been localized to neurons (Di Daniel et al., 2009; Uldry et al., 2001) and astrocytes (Uldry et al., 2001). Uldry and colleagues (2004) also found that expression of HMIT on the plasma membrane was triggered by cell depolarization, protein kinase C activation, and increased intracellular calcium concentration. Conversely, Di Daniel and colleagues found that the majority of HMIT in rat and human brain is located intracellularly, and in primary rat cortical cultures was colocalized with a golgi apparatus marker (Di Daniel et al., 2009). These results suggest that HMIT plays a role in regulating intracellular inositol and may not be involved in inositol transport into cells (Di Daniel et al., 2009).

D. *myo*-Inositol Transporters as a Function of Disease

For *scyllo*-inositol to have an effect on the AD brain, transport of *scyllo*-inositol into the CNS and within the brain is required. Evidence suggests that SMIT1 and SMIT2 are likely responsible for transport of *scyllo*-inositol into the brain, and expression has been found in the three regions of the brain affected by AD, the cortex, hippocampus, and cerebellum (Fenili et al., 2011). In the TgCRND8 AD model, SMIT1 levels in these three areas were similar when the mice were 2 months of age; however, SMIT1 in the

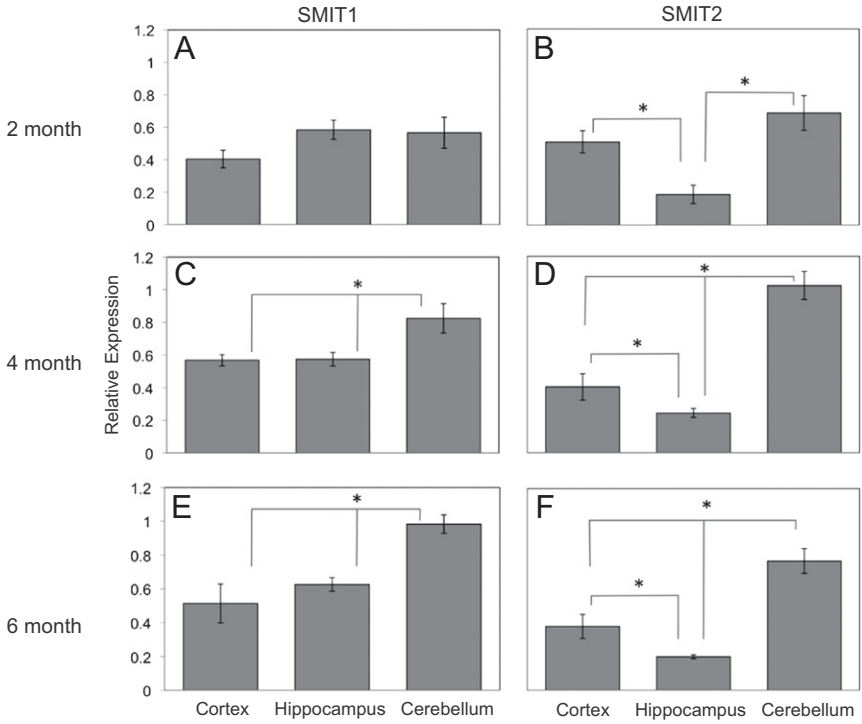

FIGURE 4 SMIT1 and SMIT2 expressions in the TgCRND8 brain as Aβ pathology advances. SMIT1 and SMIT2 expressions in the cortex, hippocampus and cerebellum in the TgCRND8 brain are not altered by age or disease progression.* $p < 0.05$.
(Reproduced from Fenili et al., 2011)

cerebellum were significantly higher than in the cortex and hippocampus at 4 and 6 months of age ($p < 0.05$). In contrast, in 2, 4, and 6 month old TgCRND8 mice, SMIT2 levels in the cortex and cerebellum were significantly higher than hippocampal levels ($p < 0.05$) (Fenili et al., 2011). These data suggest that SMIT1 and SMIT2 expression in the brain remained stable with both age and advancing Aβ pathology (Fig. 4; Fenili et al., 2011). Further it would suggest that the areas of the brain most affected by AD may have adequate *scyllo*-inositol availability even in the advanced stages of the disease.

E. Efflux

It is at least somewhat clear how *scyllo*-inositol gains entry into the brain and into cells. However, to date, there have been no studies focused specifically on *scyllo*-inositol efflux from cells. This is likely due to the fact that the function of endogenous *scyllo*-inositol has not been elucidated.

Work has been done on the efflux of the more abundant of the inositol stereoisomer, *myo*-inositol (Seaquist & Gruetter, 1998). Experiments using NT2-N neurons, primary rat astrocyte cultures, and neuroblastoma cells all suggest that *myo*-inositol efflux occurs through a volume-sensitive organic osmolyte-anion channel (VSOAC) (Isaacks et al., 1999; Loveday et al., 2003; Novak et al., 2000). *myo*-Inositol efflux is stimulated by hypo osmotic conditions and to some extent can be regulated by protein kinase C activity and intracellular Ca^{2+} levels (Loveday et al., 2003; Novak et al., 2000). While VSOAC is a nonselective Cl^- channel, it is hard to predict whether it may be involved in *scyllo*-inositol efflux.

Similar to efflux, very little is known about the degradation pathway of *scyllo*-inositol. At least some of the *scyllo*-inositol in the body is removed by direct excretion in the kidney, as *scyllo*-inositol has been identified in human urine (Yap et al., 2010; reviewed in Sherman et al., 1968). It is also possible that *scyllo*-inositol is converted into *myo*-inositol, which is then degraded through pathways in the kidney and liver. In bovine brain extracts, an $NADP^+$-dependent epimerase was identified that was capable of converting *myo*-inositol to *scyllo*-inositol and *neo*-inositol (Hipps et al., 1977). Further, in rats and rabbits it was found that *scyllo*-inositol and *myo*-inositol were able to interconvert through the intermediate *myo*-inosose-2 (Sherman et al., 1968). It should also be noted that *scyllo*-inositol levels in the CNS have been linked to *myo*-inositol levels; thus it has been speculated that *scyllo*-inositol functions as a precursor for *myo*-inositol or may be a byproduct of its metabolism (Fisher et al., 2002). Although speculative, another possibility is that *scyllo*-inositol may be degraded in the same pathway as *myo*-inositol, but without conversion to *myo*-inositol.

V. Human Clinical Trials of *scyllo*-Inositol as an AD Therapeutic

With successful results from preclinical studies, *scyllo*-inositol was approved for Phase I human clinical trial in Canada. This Phase I trial was single blinded, randomized, and placebo controlled with 12 healthy volunteers (http://www.transitiontherapeutics.com/media/news.php). Escalating doses of *scyllo*-inositol were administered to these subjects and favorable profiles of pharmacokinetics, safety, and tolerability were demonstrated. The United States Food and Drug Administration then approved a Phase I clinical trial in the United States. Overall, approximately 110 subjects participated in the Phase I clinical study and *scyllo*-inositol showed favorable safety profile and tolerability (http://www.transitiontherapeutics.com/media/news.php; Fenili et al., 2010). The pharmacokinetics of *scyllo*-inositol was assessed in the brain, CSF, and plasma in healthy volunteers. *In vivo* *scyllo*-inositol levels can be measured noninvasively using MRS (Garzone

et al., 2009). Healthy men between the ages of 24–53 were given 2000mg doses of *scyllo*-inositol BID for 10 days. MRS was used to quantify the brain concentration of *scyllo*-inositol in the gray and white matter, the posterior cingulate gyrus, and left parietal lobe, respectively. *scyllo*-Inositol levels were determined with creatine as the reference and found to increase from baseline to day 8 in all brain regions. In this study, plasma *scyllo*-inositol levels reached steady state in 5–6 days and CSF levels increased throughout the 10 days of the experiment (Garzone et al., 2009). These studies in combination demonstrated that *scyllo*-inositol is available orally and crosses the BBB to reach levels that are effective in animal models of AD.

Following safety and pharmacokinetic analyses of *scyllo*-inositol in Phase I clinical trial, *scyllo*-inositol underwent a double-blind, randomized, dose-ranging, and placebo-controlled Phase II clinical trial (Salloway et al., 2011; Fenili et al., 2010). Based on the pharmacokinetic and Phase I studies, three doses of 250, 1000, and 2000mg BID of *scyllo*-inositol were chosen. Subjects recruited for the clinical trial were between the ages of 50–85 with probable AD as determined by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association, with a Mini-Mental State Examination score between 16–26, Rosen Modified Hachinski score≤4, and a magnetic resonance imaging (MRI) scan indicating AD but healthy otherwise. The mild or moderate AD patients were randomly assigned to either the placebo group or one of the three groups of increasing *scyllo*-inositol doses, with each group consisting of 84–91 subjects. The efficacy of *scyllo*-inositol was measured by a battery of cognitive tests expressed as changes from baseline to week 78 of treatment. The Neuropsychological Test Battery (NTB) and the Alzheimer's Disease Cooperative Study-Activities of Daily Living (ADCS-ADL) were chosen as primary tests and outcome measures from the Alzheimer's Disease Assessment Scale Cognitive Subscale (ADAS-Cog), Clinical Dementia Rating-Sum of Boxes (CDR-SB), and the Neuropsychiatric Inventory (NPI) were assessed as secondary outcome measures (Salloway et al., 2011). In addition, brain ventricular volume, whole brain volume, hippocampal volume, and cortical ribbon thickness at 78 weeks of treatment were assessed using MRI. Two subsets of patients were also assessed for *scyllo*-inositol and *myo*-inositol levels in the brain using MRS and CSF. Levels of Aβ and tau were analyzed in CSF samples as biomarkers of disease progression.

One predesignated subset analysis was to determine the population pharmacokinetic properties of *scyllo*-inositol in AD patients (Liang et al., 2009). Data analyses demonstrated that plasma concentrations of *scyllo*-inositol reached steady state no later than 12 weeks of administration and concentrations were proportional to the dose. The CSF/brain concentrations reached steady state at 24 weeks and reached saturation above 1000mg BID. Overall, the patient pharmacokinetics showed moderate absorption, rapid distribution from vascular to brain with a rate-limiting step associated with a slow

clearance (Liang et al., 2009). The clearance of *scyllo*-inositol was slower in AD patients versus healthy controls, in males versus females, and as a function of renal activity (Liang et al., 2009). These data provide a pharmacokinetic model that was utilized in the analyses of exposure–response in the Phase II trial.

To determine the overall safety of *scyllo*-inositol treatment in AD patients, monitoring included assessment of treatment emergent adverse effects (TEAE), clinical laboratory tests, electrocardiograms, vital signs data, and MRI every 6 months (Salloway et al., 2011). The overall incidence of TEAE was not significantly different between the groups or as a function of ApoE ε4 genotype. However, the incidence of withdrawals was greater in the two higher doses and the number of serious adverse effects was greater in the treatment groups versus the placebo. The incidence of serious infections as well as neurologic and psychiatric adverse effects was lower in the mild AD patient population. The overall incidence of serious adverse effects was similar between mild and moderate patients. The independent safety monitoring committee analyzed the data at 48 weeks and reported more infections in the 2000mg dose and a higher incidence of death in the two highest doses (http://ir.elan.com/phoenix.zhtml?c=88326&p=irol-newsArticle&ID=1365793&highlight=; Salloway et al., 2011). Although 9 of the 10 deaths were not directly attributed to *scyllo*-inositol, those doses were electively removed from the trial. The lower dose was continued and no further deaths were reported. The only clinical laboratory measure that was reported to change was a dose-dependent decrease in uric acid. The mechanism of *scyllo*-inositol-induced infections and lower uric acid levels are unknown; however, they are under investigation (Salloway et al., 2011).

The discontinuation of the two highest doses resulted in the cognitive endpoint analyses being based on 82 placebo and 84 patients treated with 250mg *scyllo*-inositol BID (Salloway et al., 2011). Overall, none of the primary or secondary endpoints reached statistical significance. The clinical trial design included subgroup analyses of mild AD, moderate AD, ApoE ε4 carriers, and noncarriers. There were no statistically significant changes for any cognitive endpoint for the moderate AD group or effect of ApoEε4 genotype. Subgroup analysis on *scyllo*-inositol efficacy in compliant mild AD patients showed that the 250mg dose of *scyllo*-inositol treatment was significantly different compared to placebo controls as measured by the NTB *z*-score ($p = 0.007$) (Salloway et al., 2011). Although overall, including both mild and moderate patients, NTB did not show a significant difference in the 250mg treated patients compared to placebo. The effect in mild population may be the result of the NTB having greater sensitivity in evaluating mild AD patients, whereas ADAS-Cog is more sensitive for evaluating moderate AD patients. Although not significant, the Clinical Dementia Rating-Sum of the Boxes had a similar trend to that of the NTB for mild patients. These subgroup results aid in the selection of appropriate patient populations for future studies (Salloway et al., 2011.)

The use of biomarkers as primary endpoints is an area that is presently considered a vital component of drug trials for AD by physicians and is under consideration by FDA. Both imaging and CSF biomarkers were incorporated into the trial design (Salloway et al., 2011). Volumetric MRI was assessed from baseline to week 78 with ventricular volume the primary readout measure, with the inclusion of whole brain volume, hippocampal volume, and cortical ribbon thickness as exploratory measures. The well-characterized CSF biomarkers Aβx-40, Aβx-42, total tau, and phospho-tau181 were measured for changes seen between baseline and 24 weeks (steady state) or 78 weeks. *scyllo*-Inositol at 250mg dose showed a significant increase in ventricular volume compared to placebo controls although the magnitude of the change was small (Salloway et al., 2011). This finding is consistent with previous active and passive immunization trials for AD (Fox et al., 2005; Rinne et al., 2010). None of the other imaging exploratory measures were significant. The CSF biomarkers measured at 24 weeks were not significantly different from baseline; however, at 78 weeks, Aβ42 was significantly lower than placebo. These results are consistent with the CSF Aβ42 levels obtained after a 30-day treatment of TgCRND8 mouse model of AD (McLaurin, unpublished data). The efficacy of *scyllo*-inositol was not determined in this phase II trial as the power of the study was decreased due to the removal of the highest two doses. This study further established the safety profile of *scyllo*-inositol (Salloway et al., 2011). Investigation of *scyllo*-inositol as a therapeutic for AD continues as the understanding of AD evolves both pathologically and clinically.

VI. Structure–Function Analysis of *scyllo*-Inositol

Many AD clinical trials have been initiated over the last 10 years, none of which have shown efficacy as a disease modifying treatment. The failure of these compounds may be attributed to many factors beyond the lack of compound efficacy involving poor trial design, such as targeting the wrong patient population, utilizing the wrong outcome measures, or underdeveloped preclinical data thereby overestimating potential effect size. It is becoming clear that in order to design better clinical trials with an improved ability to determine disease modifying potential, one must have a detailed understanding of the preclinical measures both directly and indirectly effected by a specific compound. In light of this, the investigation into the structure–function relationship between *scyllo*-inositol and Aβ42 both *in vitro* and *in vivo* continued during clinical development.

In order to determine the binding properties that are necessary to elicit the antiaggregation activity of *scyllo*-inositol, a series of compounds substituting the hydroxyl groups with alternative functional groups were synthesized (Sun et al., 2008). The derivatives were designed to investigate the role

of both hydrogen bonding and hydrophobic interactions to the Aβ binding motif. Previous studies using inositol stereoisomers demonstrated that the all equatorial positions of the hydroxyl groups results in the most effective aggregation inhibitor and therefore was maintained in the new compounds (McLaurin et al., 2000). However, the role of each hydroxyl group as well as the hydrophobic face of the ring structure may play varying roles in efficient binding. The chemical equivalency of the *scyllo*-inositol structure allows investigation of the entire surface using substitutions at two opposing sites on the ring. Derivatives of *scyllo*-inositol were synthesized and each derivative was incubated with Aβ42 to examine inhibition of aggregation (Sun et al., 2008). Removal of one or two hydroxyl groups forming 1-deoxy-*scyllo*-inositol or 1,4-dideoxy-*scyllo*-inositol, respectively, resulted in the formation of fibers comparable to that of Aβ42 aggregation alone (Sun et al., 2008). This indicates that all hydroxyl groups are required to inhibit Aβ42 aggregation. To determine the extent hydrogen bonding contributes to this inhibition, fluorine and chlorine substitutes were synthesized. A conservative substitution with fluorine was tested because fluorine is similar in size and polarity to oxygen and it is able to act as a weak hydrogen bond acceptor, while chlorine cannot. Aβ42 incubation with 1-deoxy-1-fluoro-*scyllo*-inositol showed small amorphous aggregates, similar to the inhibitory effect of *scyllo*-inositol. However, 1,4-dideoxy-1,4-difluoro-*scyllo*-inositol was less effective (Sun et al., 2008). Single chlorine substitution showed a weaker inhibitory effect than single fluorine substitution, while the double chlorine substitution resulted in enhanced fibrillogenesis (Sun et al., 2008). To test the role of hydrophobicity, methyl groups were added, which increases the hydrophobic properties of *scyllo*-inositol and if important may enhance inhibition. 1-O-methyl-*scyllo*-inositol has an anti-aggregation effect by stabilizing protofibrillar Aβ42; whereas, two methyl substituted hydroxyl groups, 1,4-di-O-methyl-*scyllo*-inositol, showed fewer and shorter fibers than Aβ42 alone. These studies confirm the necessity for all six hydroxyl groups positioned equatorially around the inositol ring for optimal stabilization of small nontoxic Aβ42 oligomers.

The anti-Aβ-aggregation effects of 1-deoxy-1-fluoro-*scyllo*-inositol and 1,4-di-O-methyl-*scyllo*-inositol were further investigated *in vivo* because it was postulated that they may represent novel positron emission tomography (PET) imaging agents of soluble Aβ.

A. 1-deoxy-1-fluoro-*scyllo*-Inositol

1-deoxy-1-fluoro-*scyllo*-inositol is an analogue of *scyllo*-inositol with a conservative substitution of fluorine for a hydroxyl group at the C1 position of the inositol ring. In general terms, a fluorine substitution not only enhances adsorption, distribution, and metabolic stability, it can also improve protein–ligand binding (Muller et al., 2007). Incubation of

1-deoxy-1-fluoro-*scyllo*-inositol with Aβ42 resulted in small amorphous aggregates that bind the β-structure specific dye thioflavin T, similar to the parent compound *scyllo*-inositol (Hawkes et al., 2012; Sun et al., 2008). In order to determine whether the fluorine substitution would affect CNS bioavailability, 1-deoxy-1-fluoro-*scyllo*-inositol was assessed in an SMIT-1/2 transporter assay, which showed active transport suggesting oral administration would be efficient. When administered to the TgCRND8 mouse model of AD, improvement in spatial memory deficits was observed. Prophylactically, 1-deoxy-1-fluoro-*scyllo*-inositol showed a dose-dependent improvement in spatial memory with the highest dose eliciting spatial memory equivalent to that of the nontransgenic littermates (Hawkes et al., 2012). To correlate improved spatial memory with pathological markers of AD, cerebral Aβ load was analyzed. Total Aβ40 and Aβ42 levels did not change with 1-deoxy-1-fluoro-*scyllo*-inositol treatment, yet Aβ plaque load was decreased (Hawkes et al., 2012). Along with this observation, histological investigation demonstrated that microglial cells, distributed throughout the hippocampus and cortex, have processes that contain intracellular Aβ. The highest dose of 1-deoxy-1-fluoro-*scyllo*-inositol treated TgCRND8 mice showed a significant increase in Aβ-positive microglia in the hippocampus compared to untreated TgCRND8 mice (Hawkes et al., 2012). These brain-resident microglia were not associated with Aβ plaques. These results demonstrate that Aβ bound to 1-deoxy-1-fluoro-*scyllo*-inositol was taken up by microglial cells within the brain and targeted for degradation. To determine whether intra-brain degradation is a mechanism for *scyllo*-inositol effects, a similar histological investigation demonstrated a dose-dependent increase in microglial-associated Aβ after *scyllo*-inositol treatment (McLaurin, unpublished results). These findings are consistent with the decrease in CSF Aβ after *scyllo*-inositol treatment in this mouse model as well as the human Phase II clinical trial and demonstrate that *scyllo*-inositol bound Aβ is degraded within the CNS. Furthermore, the beneficial effects of 1-deoxy-1-fluoro-*scyllo*-inositol further demonstrate the potential for translation to a PET imaging agent for AD.

B. 1,4-di-O-methyl-*scyllo*-Inositol

Similar to 1-deoxy-1-fluoro-*scyllo*-inositol, *in vivo* studies with 1,4-di-O-methyl-*scyllo*-inositol yielded favorable results. *In vitro* studies showed that 1,4-di-O-methyl-*scyllo*-inositol prevented Aβ42 fibrillization by stabilizing Aβ42 protofibrils (Hawkes et al., 2010; Shaw et al., 2011). Prophylactic treatment with 1,4-di-O-methyl-*scyllo*-inositol to TgCRND8 mice also reduced escape latency in the Morris water maze test in a dose-dependent manner, indicating a rescue of spatial memory. However, treatment did not improve spatial memory to that of the nontransgenic littermates (Hawkes et al., 2010). Analysis of Aβ levels in the

brain revealed a decrease in insoluble Aβ42 levels with a concomitant increase in soluble levels. No change in Aβ40 levels was observed, while plaque load was decreased by 30%. Taken together, the decrease in insoluble Aβ and increase in soluble Aβ could be explained by the significant increase in monomeric Aβ after 1,4-di-O-methyl-*scyllo*-inositol treatment (Hawkes et al., 2010). The changes in Aβ42 but not Aβ40 suggested that this compound would not have the specificity necessary for Aβ PET imaging in AD.

C. Positron Emission Tomography Radiopharmaceuticals Based on *scyllo*-Inositol

A number of groups simultaneously have been developing PET radiopharmaceuticals based on *scyllo*-inositol (Elmaleh et al., 2010; Shoup et al., 2009; Vasdev et al., 2009). The first hurdle was to develop a synthetic scheme that renders a high radiochemical yield with a minimum of steps and maintenance of the stereospecificity. Two distinct synthetic schemes have been published that produced [^{18}F]-1-deoxy-1-fluoro-*scyllo*-inositol (Vasdev et al., 2009) and one scheme for production of 2-[^{18}F]fluoro-2-deoxy-*scyllo*-inositol (Pal et al., 2009). The highest radiochemical yield being synthesized from a very stable multifunctional precursor, 1,6;3,4-bis-[*O*-(2,3-dimethoxybutane-2,3-diyl)]-2-*O*-trifluoromethanesulphonyl-5-*O*-benzoyl-*myo*-inositol, in 80min (Vasdev et al., 2009). No matter which synthetic scheme was utilized, small animal imaging studies demonstrated very low brain penetration and high accumulation in the kidneys. Acetylation of the tracer increased brain penetrance; however, it was still below the standard for successful translation of CNS radiotracers to humans (Shoup et al., 2009). An alternate approach synthesized a series of *scyllo*-inositol derivatives attached to 2-ethyl-8-methyl-2,8-diazospiro-4,5-decan-1,3-dione to improve bioavailability (Elmaleh et al., 2010). The radiofluorination yields were high and brain uptake was greater than those reported for the PIB compound in rodents. These compounds are undergoing further investigation to determine their potential translation to AD patients.

D. Development of Novel Compounds Based on *scyllo*-Inositol

To further determine an optimal structural backbone based on *scyllo*-inositol for translation into more effective aggregation inhibitors or PET radiopharmaceuticals, establishment of a screening process was required (Shaw et al., 2011). The criterion for the development of a novel compound was stabilization of low molecular weight nontoxic oligomeric Aβ42 species, as was determined for the parent compound *scyllo*-inositol. The work plan was developed to address the effect of *scyllo*-inositol-based compounds on both structure and toxicity of Aβ. Stabilization of oligomeric Aβ species

was determined using an oligomeric specific ELISA assay, followed by structural determination using atomic force microscopy to rule out presence of amyloid fibers. It is well known that Aβ oligomers are toxic, therefore compounds that favor the stabilization of oligomers may enhance toxicity requiring toxicity assay inclusion.

Using this compound screening protocol, *scyllo*-inositol-based compounds with various substitutions were tested to optimize a backbone structure with maximal Aβ anti-aggregation/binding properties. The compounds were carefully chosen to maintain the polar periphery of the inositol ring, while allowing the exploration of potential hydrophobic binding sites. *scyllo*-Inositol was linked through either aldoxime, hydroxamate, carbamate, or amide to generate novel *scyllo*-inositol derivatives. Of the structures tested, oxime is the only linkage that positioned the phenyl substitution coplanar to the *scyllo*-inositol ring. This co-planar aromatic conformation is analogous to the aromatic structure of polyphenols, which are inhibitors of fiber formation (Bastianetto et al., 2008). Application of the polyphenol-Aβ structure–function relationship to the phenyl ring with oxime linkage to *scyllo*-inositol did not yield favorable oligomerization profile or improve inhibition of fiber formation compared to *scyllo*-inositol; thus distinguishing these flat aromatic compounds from previously reported polyphenols (Bastianetto et al., 2008). Electrospray ionization Orbitrap high-resolution mass spectrometry revealed strong binding between of the phenyl- and napthyl-oxime derivatives to Aβ42 and less stable binding between the azide derivative and Aβ42 (Shaw et al., 2011). These studies demonstrate a backbone structure that now can be utilized to develop more efficient aggregation inhibitors as well as radiopharmaceuticals for PET imaging.

VII. Inositol for the Treatment of Other Disorders

The use of inositol stereoisomers as treatment paradigms for disease has been extensively investigated. *myo*-Inositol, *epi*-inositol, and D-*chiro*-inositol have been examined for their potential therapeutic benefit to treat a variety of conditions ranging from depression to neural tube defects (reviewed in Fenili et al., 2007, 2010). However, the therapeutic properties of *scyllo*-inositol had not been investigated until 2006 (McLaurin et al., 2006). Since then other groups have corroborated the effectiveness of *scyllo*-inositol as a potential AD therapeutic. Interestingly *scyllo*-inositol has recently been proposed for potential therapeutic effectiveness in other conditions based on AD clinical trial data, involvement of Aβ in other disorders, and preclinical model studies.

The phase II clinical trial for AD demonstrated a positive clinical laboratory finding that has the potential to be translated to a number of diseases

associated with hyperuricemia (Cedarbaum, 2010). The clinical laboratory serum analyses demonstrated a *scyllo*-inositol-induced dose-dependent decrease in uric acid (Salloway et al., 2011). Hyperuricemia has been shown to be associated with, represent a risk factor for, or exacerbate certain diseases such as gout, renal disease, cardiovascular disease, metabolic syndrome, urate lithiasis, atherocleropathy, and hypertension. It was therefore proposed that *scyllo*-inositol may have beneficial effects in these disorders.

Further, the results of the phase II clinical trial, for *scyllo*-inositol treatment of AD, brought attention to the positive role of *scyllo*-inositol in reducing the development of neuropsychiatric symptoms in moderate AD patients (Salloway et al., 2011). Thus, it has been proposed that *scyllo*-inositol may have additional uses in the treatment of psychiatric disorders such as bipolar disease (http://newsroom.elan.com/phoenix.zhtml?c=88326&p=irol-newsArticle&ID=1634478&highlight=). The utility of *scyllo*-inositol in these or related disorders will need to wait further consultation with experts and appropriate proof of concept Phase II clinical trials.

Investigation into the potential utility of *scyllo*-inositol for the treatment of macular degeneration was initiated based on the role of Aβ accumulation during retina degeneration (Cruz, 2010). Macular degeneration is characterized by progressive loss of central vision and is the leading cause of blindness in the aging population. Risk factors associated with AD, ApoE genotype, and high cholesterol diet are also risk factors for macular degeneration (Cruz, 2010). In mouse models of age-related macular degeneration, *scyllo*-inositol prevented retinal defects associated with high cholesterol diet (Cruz, 2010). These preclinical mouse studies demonstrate the potential for *scyllo*-inositol effect in age-related macular degeneration; however, clinical investigations will need to be done to prove a cause-effect for this disorder.

The use of a single compound for the treatment of multiple aggregation prone proteins has been extensively investigated with considerable success in preclinical models. The potential translation of *scyllo*-inositol to other neurodegenerative disorders has also been investigated. Vekrellis and colleagues demonstrated that *scyllo*-inositol could rescue the caspase-dependent non-apoptotic death induced by overexpression of wildtype α-synuclein in a human neuronal cell line (Vekrellis et al., 2009). The potential of *scyllo*-inositol to inhibit other aggregation prone proteins will need further preclinical data in both cellular and animal models.

scyllo-Inositol and *myo*-inositol have also been investigated for effects on pentylenetetrazol-(PTZ) induced seizures in rats (Nozadze et al., 2011). PTZ is a $GABA_A$ antagonist that is used to generate animal models of epilepsy. PTZ induces convulsions similar to petit mal or absence seizures in humans. Substances able to modify the threshold for different phases of the convulsions or decrease duration of the seizure in animal models are considered to be antiepileptic (Dhir, 2012). Both *scyllo*-inositol and *myo*-inositol showed anticonvulsant properties, as they significantly reduced seizure

score, delayed the latent period for seizure onset, and decreased seizure duration (Nozadze et al., 2011). Although there were no significant differences between the *myo*- and *scyllo*-inositol treated groups it was found that *scyllo*-inositol was effective at a much lower dose compared to *myo*-inositol (Nozadze et al., 2011).

The investigation of [^{18}F]-1-deoxy-1-fluoro-*scyllo*-inositol for use in cancer as an alternate to the present standard, [^{18}F]2-fluoro-2-deoxy-D-glucose (FDG), was initiated after it was realized that peripheral tissues express SMIT1/2 (McLarty et al., 2011; Vasdev et al., 2009). Furthermore, follow up studies in breast cancer patients result in a high rate of false-positive readings as areas of inflammation have high FDG uptake and similarly high FDG uptake in the brain confounds use for detection of brain metastases (Cook, 2007). The proof of concept rodent studies utilized three human breast cancer or glioma xenograft models and turpentine-oil-induced inflammation to compare peripheral uptake of [^{18}F]-1-deoxy-1-fluoro-*scyllo*-inositol with FDG. An intracranial graft of a human glioblastoma multiforme, U-87, was successfully visualized by PET with both [^{18}F]-1-deoxy-1-fluoro-*scyllo*-inositol and FDG. The uptake of [^{18}F]-1-deoxy-1-fluoro-*scyllo*-inositol in all breast cancer and glioma xenografts was comparable or lower than FDG; however [^{18}F]-1-deoxy-1-fluoro-*scyllo*-inositol accumulated to a lesser degree in inflammation than FDG suggesting an improvement in the ability to distinguish tumor from inflammatory tissue (McLarty et al., 2011). The brain uptake associated with the intracranial glioma over normal tissue for [^{18}F]-1-deoxy-1-fluoro-*scyllo*-inositol was fivefold greater than that for FDG. Due to the enhanced contrast the glioma was more easily visualized utilizing [^{18}F]-1-deoxy-1-fluoro-*scyllo*-inositol and demonstrates a viable opportunity for imaging brain tumors. These studies are ongoing both at the mechanistic and translational level.

VIII. Conclusion

The combined preclinical and clinical data that have been generated surrounding *scyllo*-inositol treatment of Aβ-related diseases, with the predominant disease Alzheimers, have also generated great interest in this naturally occurring compound. Since the first report in 2000 on Aβ-inositol interactions, many groups have contributed to our understanding of the structure–function relationship both *in vitro* and *in vivo*. The transition to human clinical trials in a population that will most benefit from this treatment is still under investigation, although as presented, the possibilities are extensive. Further understanding of the down-stream mechanisms that are altered after removal of toxic Aβ species from the brain by *scyllo*-inositol are presently underway and may lead to new understanding of disease progression.

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Abbreviations

Acetyl-CoA	acetyl-coenzyme A
Aβ	amyloid beta peptide
ADAS-Cog	Alzheimer's disease assessment scale cognitive subscale
ADCS-ADL	Alzheimer's disease cooperative study-activities of daily living
ApoE	apolipoprotein E
APP	amyloid precursor protein
BBB	blood–brain barrier
BID	bis in die (twice daily)
CDR-SB	clinical dementia rating-sum of boxes
CNS	central nervous system
CSF	cerebral spinal fluid
DEAE	diethylaminoethyl
FAD	familial Alzheimers disease
FDA	Food and Drug Administration
FDG	[^{18}F]2-fluoro-2-deoxy-D-glucose
	N-terminus —Amino terminus
$GABA_A$	gamma-aminobutyric acid receptor A
HMIT	proton/*myo*-inositol transporter
ICV	intracerebroventricular
LTP	long-term potentiation
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NAD+	nicotinamide adenine dinucleotide (electron accepting form)
NADP+	nicotinamide adenine dinucleotide phosphate
NGF	nerve growth factor
NPI	neuropsychiatric inventory
NTB	neuropsychological test battery
NT2-N	teratocarcinoma-derived Ntera2/D1 neuron-like cells
PC-12	pheochromocytoma cells 12
PET	positron emission tomography

PIB	Pittsburg compound B
PTZ	pentylenetetrazol
SLC5A3	solute carrier family five, member three
SMIT1	sodium/*myo*-inositol transporter one
SMIT2	sodium/*myo*-inositol transporter two
TEAE	treatment emergent adverse side effects
VSOAC	volume-sensitive organic osmolyte-anion channel

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Beyond Amyloid: The Future of Therapeutics for Alzheimer's Disease

Abstract

Currently, the field is awaiting the results of several pivotal Phase III clinical Alzheimer's disease (AD) trials that target amyloid-β (Aβ). In light of the recent biomarker studies that indicate Aβ levels are at their most dynamic 5–10 years before the onset of clinical symptoms, it is becoming uncertain whether direct approaches to target Aβ will achieve desired clinical efficacy. AD is a complex neurodegenerative disease caused by dysregulation of numerous neurobiological networks and cellular functions, resulting in synaptic loss, neuronal loss, and ultimately impaired memory. While it is clear that Aβ plays a key role in the pathogenesis of AD, it may be a challenging and inefficient target for mid-to-late stage AD intervention. Throughout the course of AD, multiple pathways become perturbed, presenting a multitude of possible therapeutic avenues for design of AD intervention and prophylactic therapies. In this chapter, we sought to first provide an overview of Aβ-directed strategies that are currently in development, and the pivotal Aβ-targeted trials that are currently underway. Next, we delve into the biology and therapeutic designs associated with other key areas of research in the field including tau, protein trafficking and degradation pathways, ApoE, synaptic function, neurotrophic/neuroprotective strategies, and inflammation and energy utilization. For each area we have provided a comprehensive and balanced overview of the therapeutic strategies currently in

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preclinical and clinical development, which will shape the future therapeutic landscape of AD.

I. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive dementia. Currently the 6th leading cause of death in the United States, AD is poised to become one of the major unmet medical needs in the upcoming decade. In 2011, an estimated 5.4 million Americans were living with AD; this number is expected to grow to 7.7 million by 2030. Since advanced age is the primary risk factor for AD and aging population numbers are exponentially growing worldwide, AD will pose a severe global socioeconomic burden if no new effective therapeutics are developed. The diverse array of therapeutic avenues under development to target the complex pathophysiological mechanisms underlying the disease is summarized in the following sections.

A. Pathogenesis of Alzheimer's Disease

On a histopathological level, AD is characterized by amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques consist of insoluble extracellular deposits of amyloid-β (Aβ) protein, while NFTs are intracellular structures composed of aggregates of tau, a microtubule-binding protein (for review, see Holtzman et al., 2011). Another hallmark of AD is extensive neuronal degeneration and cell death. In the last stage of the disease, there is widespread neuronal loss that is evident as gross cerebral atrophy in multiple regions, including the temporal, parietal, and frontal lobes (for review, see Holtzman et al., 2011). As is common with other neurodegenerative diseases, there is a selective vulnerability of certain brain structures and cell types. In particular, the medial temporal lobe, including the hippocampus and entorhinal cortex (EC), appears to be the initial site of pathology. Based on magnetic resonance imaging (MRI) studies, significant atrophy is evident in these brain regions even in very mild early AD (Jack et al., 1997). While most of the neurons lost in the cortical regions are glutamatergic, certain populations of subcortical projection neurons also exhibit selective vulnerability in AD. In particular, the noradrenergic neurons of the locus coeruleus and the cholinergic neurons of the basal forebrain become dysfunctional and undergo profound neurodegeneration, relatively early in the disease (for review, see Holtzman et al. 2011). The initial observations of deficits in cholinergic neurotransmission led to the "cholinergic hypothesis" of AD, which resulted in several Food and Drug Administration (FDA)-approved drugs that inhibit acetylcholinesterase, the enzyme that breaks down acetylcholine (ACh). While these drugs

demonstrate some symptomatic efficacy, they do not significantly modify disease progression.

B. Genetic Clues for Drug Discovery

AD is categorized as either early-onset familial AD (FAD) or late-onset "sporadic" AD. In FAD, mutations in one of the three following genes, β-amyloid precursor protein (APP), presenilin 1 (PS1), or presenilin 2 (PS2), are thought to be causative of the disease. These mutations are autosomal dominant and significantly accelerate the onset of the disease state. Late-onset AD, however, is thought to be a combination of environmental and genetic risk factors, with the greatest known genetic risk factor being the presence of at least one allele of Apolipprotein E ε4 (APOEε4). Mutations in APP, PS1, and PS2, as well as *APOE* genotype, have all been shown to involve perturbation of Aβ metabolism and homeostasis (Selkoe, 2001).Genetic studies of late-onset AD point to a number of risk factor genes that encode proteins with known function in cholesterol homeostasis (notably ApoEε4, CLU (ApoJ), ABCA7, LDLR, LRP1); transmembrane proteins involved in membrane trafficking and signal transduction (SorL1, SorCS1, SorCS2, Bin1, PICALM-1, CD33, CD2AP, MS4A6A, MS4A4E) and complement factors (CR1, CRU) (Olgiati et al., 2011). Currently, our understanding of how these and other molecules relate, directly or indirectly, to pathogenesis of late-onset AD remains elusive. However, investigation of these pathways will substantially contribute to our understanding of the molecular underpinnings of late-onset AD.

C. Unique Considerations for Alzheimer's Disease Drug Discovery

While the research surrounding the mutations that contribute to AD has provided us with a strong understanding of the pathological processes dependent and independent of the Aβ hypothesis, we still do not truly understand disease causality. It is becoming clear that multiple disease targets and pathways are involved in AD pathogenesis, and the optimal time point and key disease target(s) for maximum clinical impact are still unknown. This complexity, combined with unique methodological challenges for AD, has understandably made drug discovery more challenging.

Perhaps one of the largest roadblocks in AD drug discovery is the low rate of translatability from animal studies into humans (Shineman et al., 2011). Our animal models of Alzheimer's are very poor predictors of clinical success. While AD models have fared better in terms of monitoring

target response to treatment, we don't know how these target responses translate to clinical outcomes. For example, amyloid immunotherapies in late stage clinical trials have demonstrated significant reduction of cerebral amyloid in humans, mirroring the effects seen on amyloid in preclinical animal studies. However, whether these effects on Aβ translate to cognitive benefit remains unanswered at this time. This example illustrates the utility of animal models as models of disease targets rather than the disease in its entirety. Incorporating translatable biomarkers and other novel outcome measures, and optimizing study design to reduce variability and remove bias may improve the predictability of drugs moving into the clinic (Shineman et al., 2011).

The clinical population is another hurdle in developing Alzheimer's therapeutics. Alzheimer's patients are predominately elderly, often exhibit multiple comorbidities, and are taking numerous drugs. Elderly patients differ from younger patients in their ability to metabolize drugs and their chances of developing complications. Comorbid conditions and drug–drug interactions need to be carefully monitored in clinical trials (and ultimately in clinical practice). Further, for Alzheimer's patients with memory dysfunction, a simple dosing regimen is most optimal in order to have maximum compliance.

Finally, as for all central nervous system (CNS) diseases, the blood–brain barrier (BBB) remains a hurdle in effective drug delivery. While there have been reports of BBB disruption in AD, any disruption is likely not sufficient for effective drug delivery purposes. Small molecule compounds or biologics need to be engineered to cross the BBB and reach their target in sufficient concentrations to confer biological activity. While this is an added challenge over peripheral diseases, novel drug delivery strategies (nanotechnology, medical devices, and others) and increased medicinal chemistry knowledge are helping to overcome this obstacle.

Although we are still waiting to determine the effectiveness of anti-amyloid strategies that are currently in later stage clinical trials, it is vital to the field to address non-amyloid-disease-related targets. Recent research has identified a wealth of information on pathways that contribute to the pathogenesis of AD that has enabled the identification of novel "druggable" targets. As the field begins to move away from a primary focus on amyloid and a single target intervention, these targets will become vital to a multiple-target approach, more likely to be effective. This, alongside the identification and development of new biomarker tools to diagnose AD prior to onset of clinical symptoms and to identify at risk populations, will allow for potentially disease modifying or preventative strategies. The following chapter will highlight the novel pathways that contribute to AD pathogenesis and describe the most promising therapeutic strategies currently in preclinical and clinical development.

II. Current Therapeutic Targets

A. Strategies Targeting Aβ

Overwhelming evidence points to Aβ as a disease initiator and promising drug target; however, clinical trials focused on preventing or removing Aβ accumulations have not yet demonstrated successful clinical outcomes. Aβ is generated through sequential proteolysis of the type-I transmembrane protein, APP by β-secretase and γ-secretase (for review, see Thinakaran and Koo, 2008). This amyloidogenic pathway generates Aβ peptide isoforms of 38–43 amino acids. $A\beta_{1-40}$ is the most common isoform found in AD patients, whereas $A\beta_{1-42}$ is the most amyloidogenic that forms the core of β-amyloid plaques and recruits $A\beta_{1-40}$ into these fibrillogenic cores (for review, see Glabe, 2008). The majority of therapeutic approaches described below have focused on preventing Aβ generation, disrupting its aggregation, or promoting its clearance.

1. Targeting γ-Secretase

PS1 and PS2 are known to comprise the catalytic domain of the γ-secretase complex, which is responsible for cleavage within the membrane-bound C-terminal fragment (CTF) region of APP. γ-secretase is an attractive drug target to block Aβ generation. However, γ-secretase also cleaves approximately 20 other known substrates to date, including the Notch protein involved in crucial developmental pathways (reviewed in De Strooper, 2003). Lack of substrate specificity by γ-secretase inhibitors has led to adverse events largely suspected due to inhibition of notch signaling. Eli Lilly recently released results from their Phase III clinical trial with a γ-secretase inhibitor (LY450139). The drug was able to reduce Aβ levels, but caused significant side effects and actually worsened cognition. In order to avoid these serious side effects, γ-secretase modulators are being pursued that would selectively lower Aβ42 generation without altering notch cleavage (see Table I, for overview) (Eriksen et al., 2003; Kounnas et al., 2010).

2. Targeting β-Secretase

Another potentially safer strategy to block Aβ generation is through inhibition of β-secretase (BACE1). However, accessibility of BACE1, like many other membrane proteins, is challenging. BACE1 is transported routinely between the plasma membrane and the endosomal pathway, where the more acidic environment is optimal for APP metabolism by BACE1. Recent therapeutic attempts to target BACE1 activity resulted in the genesis of cell-impermeable sterol-linked BACE-inhibitors, which attach to the plasma membrane and inhibit BACE1 activity during endocytosis (Rajendran et al., 2006). Other programs targeting BACE1 include the BACE1 inhibitors;

TABLE I Current Aβ-Targeted Therapeutics in Pivotal Phase II and Phase III Trials

Abeta Targeted Therapeutic Strategies

Drug name	*Target*	*Investigator*	*Phase*
Abeta immunotherapies			
Sloanezumab	Monoclonal antibody	Eli Lilly and Company	III
Bapineuzumab	Monoclonal antibody	Pfizer/Elan/J&J	III
ACC-001	Immunogenic Abeta 1-6	Janssen	II
MABT5102A	Monoclonal antibody	Genentech	II
Gantenerumab	Monoclonal antibody	Hoffman-La Roche	II
AFFITOPE AD02	Abeta 1-6 from B cell	Affiris AG	II
CAD 106	Immunogenic Abeta 1-6	Novartis	II
Crenezumab	Monoclonal antibody	Hoffman-La Roche	II
Gamma-Secretase inhibitors and modulators			
LY450139	GSM	Eli Lilly and Company	III—Failed, adverse effects
NIC5-15	GSI	Department of Veterans Affairs	IIB
CHF5074	GSM	Chiesi Pharmaceuticals Inc.	II
BSM-708163	GSI	Bristol-Myers Squibb	II
Regulators of APP Metabolism and Abeta Aggregation			
ELND005	Inhibits Abeta oligomer formation	Elan/Transition Therapeutics	II/III
PBT2	Attenuates metal–Abeta interaction	Prana/ADDF	II
EVP-6124	α7 nAChR	EnVivo Pharmaceuticals, Inc.	II
ST101	Induces 17-kDa APP fragment cleavage	Sonexa Therapeutics, Inc.	II

SCH745966 (Merck) and CTS21166 (Astellas/Comentis) and BACE1 monoclonal antibodies engineered to cross the BBB via the transferrin receptor (Genentech) (for review see Lane et al., 2011).

3. α-Secretase

APP can also be processed via a constitutive, non-amyloidogenic pathway in which APP is proteolysed within the Aβ sequence by α-secretase (ADAM10, ADAM17). This cleavage results in the secretion of α-APP N-terminal domain (sAPPα) and an 83-amino-acid membrane-bound CTF (C83-CTF) (for review, see Small and Gandy, 2006). Interestingly, sAPPα has reported neuroprotective effects and enhances dendrite outgrowth (for review, see Zhou et al., 2011). Overexpression of ADAM10 in human APP

transgenic mice resulted in an increase in sAPPα production, where as expression of catalytically inactive ADAM10 resulted in increased Aβ plaques and cognitive deficits (Postina et al., 2004), suggesting that design of specific allosteric enhancers/regulators of ADAM10 may represent one potential therapeutic avenue for design of AD therapeutics (Fig. 1).

4. *Anti-Amyloid-β Aggregation*

For decades, it was assumed that insoluble fibrillar Aβ (plaques) were the most pathogenic Aβ assembly; however, recent evidence indicates that accumulation of Aβ into soluble oligomeric Aβ (oAβ) species may be more detrimental to neuronal function and may be responsible for spatial memory deficits in rodents (Gandy et al., 2010; Lesne et al., 2006). Electrophysiological studies have shown that addition of oAβ to hippocampal slices results in inhibition of long-term potentiation (LTP), a cellular model of learning and memory (for review, see Fandrich, 2012). These results were corroborated *in vivo* via deficits in learning and memory performance following injection of oAβ directly into the hippocampi of living rats (Shankar et al., 2007). Taken together, it becomes evident that targeting Aβ aggregation may represent a viable therapeutic strategy; however, pertinent questions arise as to which assemblies ought to be targeted. While early work on Aβ aggregation inhibitors has not met with success (tramiprostate, Neurochem), groups are now working on specifically targeting oAβ through immunotherapy (described later) or by blocking oAβ's interaction with neuronal membranes (see "synaptic plasticity and cognition").

Interestingly, it has been suggested that interactions between Cu and proteins involved in AD may regulate the aggregation of Aβ (for review, see Kaden et al., 2011). Moreover, treatment with clioquinol or its second-generation analogue (PBT2, Prana Biotechnology) rapidly restored cognition in APP transgenic mice and was associated with decreased interstitial Aβ (Faux et al., 2010). In a double-blind and placebo-controlled 12-week Phase-IIa study of PBT2, patients had a dose-dependent reduction of CSF Aβ, and demonstrated significant improvement in two tests of executive function (Lannfelt et al., 2008). While these studies suggest that there is some hope for anti-Aβ therapies in the AD therapeutic landscape, it remains to be determined whether directly targeting Aβ represents a viable option for developing disease intervention strategies.

5. *Aβ-Immunotherapy*

Immunotherapies targeting Aβ can lower fibrillar amyloid load by up to 25% after 75 weeks of treatment though, so far this success in amyloid-lowering has not been associated with obvious cognitive benefit in AD patients (Gandy et al., 2010; Holmes et al., 2008; Rinne et al., 2010). This may relate, in part, to a lack of distinction between monomeric and multimeric assemblies by the prototypical monoclonal anti-Aβ antibodies

employed in these studies (i.e. Bapeneuzumab), where immunotherapies targeting toxic structural epitopes, such as specific oligomeric species, may be more therapeutically relevant (for a detailed discussion on immunotherapy, see Section I.G).

Taken together, safety concerns with Aβ targeted therapies remain controversial and suggest that total inhibition of Aβ generation may be less advantageous in therapeutic reality. Timing of Aβ intervention is also a critical consideration, as we know that Aβ accumulates during the decades prior to clinical presentation. The inclusion of amyloid imaging and CSF Aβ measurements in future trials of Aβ-lowering therapies should enable standardization of subjects according to stage of neuropathology rather than the current practice of standardization according to cognitive status and thereby hopefully reduce variability in clinical trials.

B. Tau-Targeted Therapeutics

While tau was initially hypothesized to be a passive marker of late stage disease, a plethora of new research implicates tau as an active initiator of cell death and thus an important drug target for AD. NFTs composed of hyperphosphorylated tau protein are present in a number of neurodegenerative diseases in addition to AD. These neurodegenerative diseases are referred to as tauopathies and include AD, frontotemporal dementia (FTD) with parkinsonism (linked to chromosome 17; FTDP-17), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), as well as juvenile lysosomal storage diseases (LSDs) such as Nieman–Picks type C disease (for review, see Morris et al., 2011). While tau hyperphosphorylation and tangle formation are common between these different tauopathies, pathology differs in the conformation and localization of tau deposition, as well as the expression of alternatively spliced tau isoforms.

The mechanism of how tau pathology relates to the overall disease process is still largely unclear. Tau is a predominantly neuronal protein whose main function is to stabilize microtubules, ensuring viable transport of cargo to neuronal processes. Hyperphosphorylation of tau leads to detachment from microtubules, microtubule destabilization, and breakdown of the neuronal cytoskeleton. Hyperphosphorylated tau accumulates into abnormal twisted fibers, called paired helical filaments (PHFs), that form higher-order structures termed neurofibrillary tangles, or neuropil threads in neurons and other types of brain cells (for review, see Holtzman et al., 2011). For many years, it was assumed that tau tangles represented dying neurons and were a consequence, rather than a cause, of disease progression. The degree of tangle pathology is tightly correlated with cell loss and clinical disease severity (Braak & Braak, 1997), leaving some to hypothesize that tau tangles were tombstones depicting dead neurons.

The strongest evidence for abnormal tau metabolism as a contributing factor in neurodegeneration, rather than a passive bystander, occurred when tau mutations were identified in families with FTDP-17 (Hutton et al., 1998). To date, almost 40 mutations have been identified in the *MAPT* gene. Most mutations cluster around the microtubule binding domain (referred to as the repeat region), while others affect mRNA splicing. Post-transcriptional splicing of the *MAPT* gene generates up to six isoforms of gene product in the human brain. Inclusion or exclusion of Exon 10 results in two classes of tau isoforms: tau with three repeat regions (3R) or four repeat regions (4R). Most disease-associated mutations that alter splicing affect the ratio of 3R to 4R tau (for review, see Wolfe, 2009). To date, despite the fact that one of the key pathological hallmarks of AD is NFTs, no genetic mutations have been detected in the *MAPT* gene in AD. This is, in part, the reason why the field has focused primarily on Aβ as a drug target for AD, and why tau-focused drug therapies lag behind Aβ therapies in development.

The discovery of these tau mutations linked to tauopathies, however, does not clarify the debate as to whether tau dysfunction results from a loss of tau's normal function to stabilize microtubules, or from toxic gain-of-function as a result of tau tangle accumulation. In support of the loss of function hypothesis, tau knock-out mice develop cognitive and motor deficits as they age (Ikegami et al., 2000). On the other hand, animal models engineered to overexpress tau with familial mutations linked to FTDP-17 show age-dependant tau phosphorylation, tangle formation, and neuronal cell loss, perhaps demonstrating a causal link between tangle formation and toxicity (Gotz et al., 2007). Further, recent evidence indicates that hyperphosphorylated tau mislocalizes to the dendrites in disease and may directly alter synaptic function (Hoover et al., 2010). Given the evidence presented above, it is likely that toxicity is due to both a loss of tau's normal function, as well as a toxic gain-of-function.

The interaction between Aβ and tau has been difficult to understand. Recent reports indicate that tau may mediate Aβ-induced neuronal dysfunction. While it seems likely that these two pathological pathways interact with each other in some way, more research is needed to fully understand this relationship. New therapeutics that target tau pathology in the many ways described later will help to address whether blocking tau pathology will impact amyloid pathology and/or if tau drugs will block the downstream pathological events hypothesized to be initiated by Aβ (for overview of tau strategies, see Table II).

As the amount and distribution of tangle pathology has been correlated with neuronal cell death and clinical disease severity (Braak & Braak, 1997), preventing tau aggregation and tangle formation may prevent cell death from occurring. Currently, investigators are working on a number of therapeutic strategies to disrupt tangle formation and prevent tau-toxicity in AD,

TABLE II Tau-Focused Therapeutic Strategies

Hyper-phosphorylation	GSK-3beta Inhibitors
	Broad-range kinase inhibitors
	PP2A activators
	O-glycNACase inhibitors
	Acetylation modulators
Clearance	HSP90 inhibitors
	Autophagy/proteasome Activators
	Tau immunotherapy
Aggregation	Aggregation inhibitors
Loss-of-function	Microtubule stabilizing agents

including directly disrupting tau aggregation, promoting pathological tau clearance, and/or targeting numerous pathways that directly or indirectly regulate tau phosphorylation (Dickey et al., 2006; Iqbal & Grundke-Iqbal, 1998).

1. Posttranslational Modification

Tau hyperphosphorylation results in disassociation of tau from the microtubules and is thought to promote tau aggregation. There are over 85 reported sites of tau phosphorylation, with some sites thought to be earlier events while other sites occur with more advanced stages of pathology (for review, see Dolan and Johnson, 2010). Numerous kinases have been implicated in tau phosphorylation, including GSK-3β, CDK5, MAPKs, PKA, Akt, PKC, CAMKII, and so on. Cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK-3β) have both been implicated as early tau kinases in catalyzing the over-phosphorylation of tau at specific motifs (serine- or threonine-proline) promoting its aggregation and formation of NFTs (Lopez-Tobon et al., 2011; Medina, Garrido, & Wandosell, 2011).

GSK-3 inhibitors were one of the first categories of drugs to progress into clinical trials for AD. GSK-3 has been shown to phosphorylate tau protein in human neuronal cells (Hong & Lee, 1997). Studies have also shown that increased GSK-3 activity may regulate Aβ generation by increasing γ-secretase activity (Phiel et al., 2003). Therefore, it is an attractive drug target for disease modification. GSK-3 exists in two isoforms, α and β, with each isoform reported to have independent functions related to Alzheimer's pathology, despite 98% homology. GSK-3 is known to have numerous substrates in addition to tau and to affect many downstream pathways including the cell cycle, metabolism, and survival signaling pathways. Lithium, which possesses GSK-3 inhibitory activity as one of its many functions, entered a number of clinical trials in AD patients. While Lithium has been shown to slow cognitive decline in patients with amnestic mild cognitive impairment (MCI) (Forlenza et al., 2011), other trials have not been as

positive (Hampel et al., 2009). Despite some of these promising findings, lithium is not a realistic treatment option for elderly Alzheimer's patients, as therapeutic doses are not well tolerated, providing a very narrow window for treatment. Therefore novel, specific small molecule inhibitors of GSK-3 are also under development for AD and have shown efficacy in animal models of AD (for review, see Medina et al., 2011).

While results may be encouraging on the development of kinase inhibitors for AD, specificity across other important biological pathways and toxicity remain a concern. In light of the toxicity concerns of inhibiting one kinase specifically, another strategy that is growing in popularity is to take a broader network approach to a disease target. Inhibiting multiple kinases at lower levels could reduce side effects, while still significantly inhibiting tau phosphorylation. Such an approach with a broad-range kinase inhibitor derived from a natural product, such as K252a, demonstrated reduction of pathological tau species and improvement on related behavioral outcomes (Le et al., 2006).

A complementary strategy to targeting tau hyperphosphorylation is to increase the activity of tau phosphatases. Protein phosphatase 2A (PP2A) has been shown to dephosphorylate tau *in vitro* and is associated with tau in neurons within the human AD brain (reviewed in Voronkov et al., 2011). Therefore, investigators are developing strategies to activate PP2A. High dose of sodium selenate, for example, has been shown to mitigate tau pathology through PP2A in Alzheimer's mice (van Eersel et al., 2010). Sodium selenate could potentially be applied clinically, although safety could be a concern at high doses. Other small molecule approaches to PP2A activation are still in the preclinical stages. PP2A is regulated by a PP2A-specific methyltransferase (PME-1; Xing et al., 2008). Signum Biosciences has identified a compound that increases PP2A activity by regulating its methylation and are persuing this approach preclinically (http://www.signumbiosciences.com/).

In addition to hyperphosphoylation, tau protein can be posttranslationally modified by a number of other moieties, including glycosylation and acetylation, which can both alter tau pathogenicity. O-glycosylation is inversely correlated with tau hyperphosphorylation, therefore, preventing the removal of these modifications could reduce tau hyperphosphorylation. O-glyNACase inhibitors (which prevent the removal of O-glycosylation from tau) have gone through preclinical development (Yuzwa et al., 2008). This work led by Dr. Vocadlo was spun out into a biotechnology company, Alectos Therapeutics, which struck a deal with Merck in 2010.

Acetylation was recently identified as a pathological posttranslational modification that may alter tau's ability to interact with microtubules and opens up the possibility that inhibition of tau acetylation, or activation of tau deacetylases, may be viable therapeutic avenue(s) for the future (Cohen et al., 2011; Min et al., 2010). Although, Cohen et al. suggest that acetylation may be a common mechanism regulating multiple microtubule binding proteins specificity for tau will need to be carefully monitored.

2. Tau Conformation

Clearing conformationally distinct tau species that are specific for AD has emerged as a new therapeutic strategy. HSP90 inhibition may specifically target pathological conformations of tau for degradation via the proteosome (Dickey et al., 2006). A number of groups are working to develop novel HSP90 inhibitors for neurodegenerative disease that can cross the BBB. Compounds that induce proteosomal activation, as well as autophagic/lysosomal degradation, are also under investigation for their ability to clear tau aggregates (see Section II.C).

Tau conformation can also be modified by the enzyme prolyl isomerase (Pin1), which affects its ability to interact with microtubules and be phosphorylated by kinases. The tau protein contains numerous proline residues in its microtubule binding domain that can exist in one of two interchangeable conformations (*cis*/*trans*). This *cis*/*trans* conformation, accelerated by Pin1, alters the ability of kinases and phosphatases to interact with tau. Pin1 restores the ability of tau to bind microtubules and can promote microtubule assembly *in vitro* (Lu et al., 1999). Pin1 is also elevated in AD patient brains, correlates with degree of pathology, has been implicated in altering APP processing, and may also have other functions related to AD. Therefore, enhancing Pin1 function or preventing its loss of function has the potential to promote proper tau function and microtubule stability in disease.

3. Immunotherapy

Numerous groups are working on immunotherapy approaches to clear pathological tau from the brain. While these programs are still in preclinical stages, the most advanced programs target pathological conformations of tau. Active immunization with synthetic phosphorylated tau peptides reduces tau aggregation and slows the development of NFT accumulation in mouse models of tauopathy (Asuni et al., 2007; Boimel et al., 2010). While the mechanism of clearance in still under investigation, evidence suggests stimulation of lysosomal degradation of tau. New findings on tau spreading from cell to cell and disease transmission have helped stimulate new hypotheses on how tau immunotherapy may work (Clavaguera et al., 2009; de-Calignon et al., 2012; Liu et al., 2012). Immunotherapy could potentially be targeting extracellular tau and stimulating endosomal uptake and lysosomal degradation. Given concerns regarding safety with active immunizations, tau passive immunotherapy approaches with monoclonal antibodies are also under development for AD.

4. Aggregation

Tau aggregation inhibitors have been explored as a therapeutic avenue for some time, although, like Aβ-aggregation inhibitors, these have been

difficult to develop. A number of classes of molecules have been identified that inhibit tau aggregation *in vitro* and groups are working to improve compound properties for *in vivo* delivery (Bulic et al., 2009; Johnson et al., 2010). These chemical classes include cyanine dyes such as thioflavin S, phenothiazine, anthraquinonoes, phenylthiazolylhydrazides, benzothiazoles and rhodanine-based compounds. While these programs are still in early-stage preclinical development, one phenothiazine compound, methylene blue, has been progressing though AD clinical trials. Methylene blue was originally used in the clinic to treat malaria and has been shown *in vitro* to disrupt tau aggregation (Wischik et al., 1996). Methylene blue is now being developed by TauRX (http://www.taurx.com/), and results from a Phase II clinical trial presented at the International Conference on AD in 2008 showed encouraging clinical efficacy (Gura, 2008). Methylene blue, however, has many other functions besides just disrupting tau aggregation (such as improved mitochondria function and disaggregation of other disease proteins and neuroprotection). Therefore, while methylene blue may be a promising drug, the therapeutic mechanism of action is still unclear and specificity may be a concern.

Other drugs in the clinical pipeline target the loss of function induced by tau hyperphosphorylation and aggregation, namely microtubule instability. Microtubule stabilizing agents are under development for neurodegenerative disease application. Allon Pharmaceuticals is developing a small peptide, davenutide (AL-108), that is thought to be neuroprotective by stabilizing microtubules, although it may have other functions as well (Matsuoka et al., 2007). Davenutide has been shown to protect neurons from Aβ-induced insults (Gozes & Divinski, 2004). The drug paclitaxel, an anticancer agent, has also been shown to stabilize microtubules, reverse axonal transport deficits and improve locomotor function in tau transgenic mice (Zhang et al., 2005). Paxlitaxol, however, has limited brain bioavailability. An alternative microtubule stabilizing agent with improved brain penetration is Epothilone D. Treatment of PS1Δ9 tau transgenic mice with Epothilone D improved microtubule density and axonal integrity and boosted cognitive behavioral outcomes (Brunden et al., 2010).

In summary, there are many approaches in preclinical development to target tau production, pathological accumulation, and cellular dysfunction induced by tangle formation, yet few of these approaches have reached human clinical trials (for summary of approaches see Table II). Over the next few years, as more of the therapeutic strategies reach human testing, we will begin to understand how tau-targeted interventions will play into the therapeutic landscape for AD. With improvement in tau CSF biomarkers and the development of tau ligands for neuroimaging, target engagement for tau-based therapies will be able to be more readily assessed, improving the interpretability of tau clinical trials moving forward.

C. Protein Sorting and Degradation Pathways as Therapeutic Strategies

Cellular homeostatic mechanisms such as protein trafficking and turnover are crucial to proper neuronal function. Perturbations in these pathways have been directly linked to AD and may specifically antagonize AD pathological cascades. Indeed several of the top 15 genetic risk factors for late-onset AD (http://www.alzgene.org/), identified in genome-wide association studies (GWAS), are implicated in or have now been shown to regulate the intracellular trafficking of APP and/or the secretases.

One of the earliest reported changes in AD is in the process of clathrin-mediated endocytosis (CME) and endosomal trafficking, where Aβ is most readily generated (for review, see Small and Gandy, 2006). Numerous proteins involved in this pathway have been implicated in AD, including the adaptor proteins involved in CME (PICALM1 and Bin 1), the rab GTPases (Rab5, Rab7) that regulate early endosome, late endosomal trafficking, and the retromer complex (Vps35, Vps26) and its receptors (sortilin family) that regulate endosome to TGN trafficking of APP and BACE1. A wealth of evidence now implicates dysregulation within the endosomal pathway during the onset of AD pathology (Fig. 1).

1. Endosomal Dysfunction

In preclinical AD, enlarged endosomes are evident prior to NFT formation, cerebral vascular amyloid deposition, and clinical symptoms (Cataldo et al., 1997, 2000, 2004). Upregulation of Rab5, a positive modulator of endocytosis, and rab7, a regulator of late endosome-lysosome transport, has been reported in the cholinergic forebrain and in the CA1 region of the hippocampus during the preclinical stages of AD (Ginsberg, et al., 2010a; Ginsberg et al., 2010b; Ginsberg et al., 2011). Strong evidence suggests that APP and BACE1 colocalize with rab5 and rab7 positive endosomes and accumulation of Aβ occurs within these compartments. Rab5 and Rab7 have also been implicated in nerve growth factor (NGF) and brain-derived growth factor (BDNF) signaling (Deinhardt et al., 2006; Liu et al., 2007; Saxena et al., 2005a; Saxena et al., 2005b), with endosome dysregulation being proposed to contribute to long-term deficits in hippocampal neurotrophic signaling (see Section II.F; Ginsberg et al., 2010a; Ginsberg, et al., 2010b; Ginsberg et al., 2011). In fact, upregulation of rab 5 paralleled decreased expression of the BDNF receptor, TrkB in the hippocampus of AD patients (Ginsberg, et al., 2010a).

Upon exit from the late endosome, cargo is either trafficked via the lysosomal pathway for degradation or recycled back to the trans-Golgi network (TGN). Several proteins implicated in retrograde recycling to the TGN have been implicated as risk factors in late onset AD, FTD, and Parkinson's disease (PD). The mammalian retromer complex consists of two subunits,

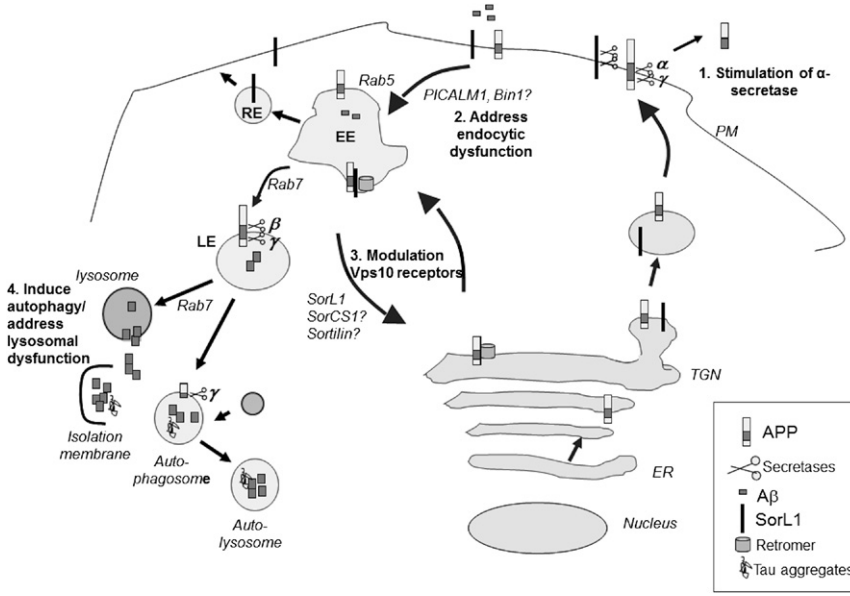

FIGURE 1 Therapeutic strategies related to APP and tau intracellular trafficking and degradation. APP and SorL1 are trafficked from the *trans-Golgi network (TGN)* to the plasma membrane (PM). On route to and at the PM, APP and SorL1 are cleaved by the α- and γ secretases. Unprocessed APP and SorL1 are subsequently re-internalized into the endosomal pathway. SorL1 and the retromer function in retrieval of APP from the early endosomal (EE) compartments to the *TGN*. SorCS1 also functions as a retromer receptor for APP retrograde trafficking. APP molecules that reach the late endosome (LE) undergo processing by β and γ secretases resulting in Aβ generation. Accumulation of Aβ within these compartments leads to lysosomal leakage resulting in the intracellular accumulation of Aβ peptides. Macroautophagy responds through the generation of an isolation membrane around protein aggregates (i.e., Aβ, tau). Subsequent formation of autophagosomes leads to autophagosome–lysosome fusion and autolysosome biogenesis leading to degradation. Therapeutic strategies relevant to these pathways include: (1) Stimulation of the α-secretase (ADAM10, ADAM 17), resulting in increased sAPPα and decreased amyloidogenic processing of APP. sAPPα also has reported neuroprotective effects. (2) Modulation of the Vps10 receptor expression levels or activity, potentially leading to increased APP retrograde trafficking, reduced Aβ deposition, and reduced endosomal dysfunction. This strategy would also promote neutrophin signaling that is disrupted with endosome dysfunction. (3) Stimulation of autophagy and lysosomal degradation. This proposed therapeutic strategy would clear Aβ and tau protein aggregates early in disease progression.

the sorting nexin (SNX) proteins (SNX1 and 2) and the vacuolar protein sorting (Vps; Vps26-Vps29-Vps35) protein complexes (for review, see McGough and Cullen, 2011). The core components of the Vps subcomplex, Vps35 and Vps26, have now been shown to be decreased in the brains of AD patients (Muhammad et al., 2008) and *in vitro* and *in vivo* models

demonstrate that Vps35 haploinsufficiency is sufficient to influence BACE activity and Aβ production (Wen et al., 2011).

2. *Sortilin Family of Vps10 Receptors*

Several members of the sortilin family of Vps10 receptors have been implicated as retromer receptors and have been now linked to late onset AD (Liang et al., 2009; Rogaeva et al., 2007). The sortilin family comprises five receptors: SorL1, SorCS1, SorCS2, SorCS3, and sortilin that are characterized by an N terminal Vps10 homology domain. SorL1 (SorLA/LR11) was the first member of this family to be identified in GWAS studies as a risk factor for late-onset AD (Liang et al., 2009; Rogaeva et al., 2007) and is downregulated in the brains of late-onset AD and MCI patients (Dodson et al., 2006; Sager et al., 2007; Scherzer et al., 2004). Through *in vitro* and *in vivo* studies, SorL1 was shown to directly interact with APP and modulate Aβ generation (Andersen et al., 2005; Nielsen et al., 2007; Offe et al., 2006; Schmidt et al., 2007), reportedly through a direct interaction with the retromer (Fjorback et al., 2012). It is proposed that SorL1 functions as a cargo receptor for the retromer, trafficking APP away from the endosomal system and an environment of high BACE activity to the TGN (Fig. 1; Fjorback et al., 2012b; Nielsen et al., 2007). Reduced expression or activity of Vps35 and/or SorL1 reportedly increases Aβ generation (Muhammad et al., 2008; Nielsen et al., 2007; Offe et al., 2006). Recent work, however, even now implicates SorL1 in the direct regulation of APP proteolysis through the regulation of APP oligomerization (Schmidt et al., 2011).

A second member of the sortilin family, SorCS1, originally identified as a risk factor for type 2 diabetes melitus (T2DM) (see Section II.H), was subsequently identified in GWAS studies as a risk factor for late onset AD (Liang et al., 2009). SorCS1 expression levels are decreased in the patient's brain with AD (Reitz, Tokuhiro, et al., 2011) and variations in intron 1 of SorCS1 have now been associated with memory changes in AD patients (Reitz, Lee et al., 2011). *In vitro* and *in vivo* studies demonstrate that SorCS1 is a regulator of Aβ generation (Lane et al., 2010; Reitz, Tokuhiro, et al., 2011), with evidence to suggest this function is mediated in part by regulation of APP retrograde trafficking through an interaction with the Vps35 subunit of the retromer (Lane et al., 2010). A third member of sortilin family has also been implicated as the retromer receptor for BACE (Finan et al., 2011). However, it is not yet clear how cargo selection between the different retromer receptors occurs. Sortilin has additionally been linked to FTLD, providing a link between Tar DNA-binding protein (TDP) 43 and progranulin pathology (Hu et al., 2010). Finally, SorCS2, a fourth member of the sortilin family, was also recently genetically associated with late onset AD (Reitz, Tokuhiro, et al., 2011).

Independent of these established roles of the Vps10 family in sorting of proteins that is central to the accumulation of protein aggregates in AD, PD,

and FTLD, they also function in neurotrophin signaling (see Section II.F). Sortilin-p75^{NTR} interaction is required for proNGF activation of the pro-apoptotic cell death pathway (Nykjaer et al., 2004), and a role for SorCS2 was described in initiating acute collapse of growth cones in hippocampal neurons (Deinhardt et al., 2011). Expression of the sortilin family members is regulated in part through BDNF activation of ERK and, to date, BDNF modulation of Aβ levels was demonstrated to be dependent on the presence of SorL1 (Bohm et al., 2006). Small molecules that regulate expression or function of the Vps10 family of receptors or of the core retromer components are therefore attractive therapeutic targets although their relative "druggability" still remains to be evaluated. Targeting these pathways, however, would create the possibility of modulating the generation and accumulation of protein aggregates, preventing endosome dysfunction and promoting neurotrophin signaling pathways, all of which have clear implications for a number of neurodegenerative diseases.

3. *Lysosomal Function*

Modest changes have been demonstrated in sphingolipid metabolism in AD, amyotrophic lateral sclerosis (ALS), and PD. Several studies now demonstrate a relationship between sphingolipid and sphingomyelin metabolism and Aβ generation (for review, see Mielke and Lyketsos 2010).

It has long been recognized that Aβ exists in a complex with gangliosides. Ganglioside binding to Aβ on neuronal membranes leads to the generation of Aβ with an altered conformation that promotes aggregation and fibril formation (for review, see Yanagisawa (2011)). Several LSDs including Sandhoff disease and Niemann–Pick type C disease exhibit pathological similarities to AD, including intracellular Aβ accumulation and intracellular tau accumulation (for review, see Yanagisawa, 2011). While enzyme replacement strategies have previously been developed for Fabry's disease and Gaucher's disease, these strategies are unsuccessful at improving CNS manifestations. However, Amicus therapeutics (http://www.amicustherapeutics.com/) is currently developing a novel class of BBB permeable pharmacological chaperones that stabilize and increase cellular levels of target enzymes to address this need. Their Alzheimer's program is still in its preclinical stages and aims to test a lead compound in cerebral amyloidosis, as an orphan indication with respect to AD (for review, see Lane et al., 2011).

4. *Autophagy*

Macroautophagy is fast becoming an area of interest in the development of therapeutics for multiple neurodegenerative diseases. Dystrophic neurons in AD contain an excess of electron dense autophagosomes and autolysosomes in neocortical and hippocampal pyramidal neurons (Nixon et al., 2005). Evidence suggests that the dysfunction of the lysosomal and/or autophagic pathways play an important role in the accumulation and/or clearance of protein aggregates.

The development of therapeutics that increase autophagic activity is growing in popularity. In cell models, drosophila, and mouse studies, upregulation of autophagy through inhibition of the mTOR (mamalian target of rapamycin), or equivalent pathways, reduced levels of both mutant Huntington and tau (for review, see Harris and Rubinsztein, 2011). While rapamycin is known to induce autophagy, it should be noted that it targets multiple signaling pathways and its effects on protein aggregation cannot therefore be solely attributed to stimulation of autophagy.

Therapeutics that target autophagy independent of mTOR have also shown to be effective inducers, or enhancers of autophagy. Compounds that inhibit inositol synthesis leading to decreased levels of IP3 were demonstrated to induce autophagy and reduce accumulation of protein aggregates in cell and animal models (for review, see Harris and Rubinsztein, 2011). Furthermore, recent data generated by Yu and Duff (Columbia University) demonstrated that treatment of *ex vivo* slice cultures and tau transgenic mice with trehalose, a nonreducing dissacharide stimulated autophagy, reduced tauopathy and improved cognitive performance (for review, see Lane et al., 2011). Although these data support the hypothesis that induction of autophagy is a valid therapeutic mechanism for clearance of protein aggregates, a body of data in the field supports the hypothesis that downstream autolysosome dysfunction is a key contributing factor to autophagic dysfunction and therefore inducing autophagy without addressing this dysfunction would not be sufficient (for review, see Nixon & Yang, 2011).

Autophagic vacuoles are rich in APP, APP βCTF, and γ-secretase activity. In fact, upon induction of autophagy, 20% of γ-secretase activity has been shown to occur within autophagic vacuoles (AV) (Yu et al., 2004, 2005). One hypothesis is that early autophagic induction results in the accumulation of AVs (marked by increased LC3-II), which promotes Aβ production. Accumulation of Aβ within these compartments is then proposed to contribute to further disruption of autophagosome maturation and/or autolysosome function, leading to decreased autophagic/lysosomal clearance (Yu et al., 2005). The hypothesis that autolysosome dysfunction is the key contributing factor to progression of the disease is additionally supported by observations in genetic models where deletion of cystatin B, an endogenous inhibitor of lysosomal cysteine proteases, enhanced clearance of Aβ and rescued cognitive deficits in the CRND8 model (Yang et al., 2011b). Familial mutations in PS1 were also demonstrated to directly affect lysosomal function, where FAD PS1 mutations prevent the correct post-translational modification of V0-ATPase preventing its trafficking to the lysosome (Lee et al., 2010). These data provide a direct link in FAD to impaired clearance of protein aggregates due to lysosomal dysfunction (Lee et al., 2010).

In terms of the "druggability" of this pathway, as discussed earlier, it is becoming more evident that simply inducing autophagy may not be sufficient

to promote the clearance of protein aggregates. The deficiency of autophagic/lysosomal clearance both in FAD cases and in late-stage late onset AD cases (for review, see Yang et al., 2011a) suggests that therapeutic intervention targeting the autophagic pathway may be insufficient to correct these deficits, if targeted upstream of lysosomal clearance. Further work in the field is required to determine if induction of autophagy following the arrest of autophagic/lysosomal clearance would actually result in hyperaccumulation of toxic protein aggregates, exacerbating disease progression (Fig. 1).

In conclusion, evidence from multiple genetic and cell biology studies demonstrate a central role for proteins involved in protein trafficking and degradation in the generation and intracellular accumulation of protein aggregates common to a number of neurodegenerative diseases. However, the "druggability" of these pathways and the timing of treatment remain to be established. These are important considerations when targeting trafficking and degradation pathways, since it is unclear whether targeting these pathways following the development of pathology will be sufficient to alter disease progression.

D. ApoE4-Targeted Therapeutics

The identification of apolipoprotein E (ApoE)-Aβ complexes in the CSF prompted the identification of the ApoE ε4 allele as the first genetic risk factor for late onset AD (Corder et al., 1993; Strittmatter et al., 1993). The most common single-nucleotide polymorphisms (SNPs) in the *APOE* gene result in the *APOE* ε2, *APOE* ε3 and *APOE* ε4 alleles that result in the following six different genotypes; ε2/ε2, ε3/ε3, ε4/ε4, ε2/ε3, ε2/ε4, ε3/ε4. The three isoforms differ only at two residues: 112 and 158, with ε2 encoding cysteines at 112 and 158; ε3, a cysteine at 112 and arginine at 158; and ε4, arginine residues at 112 and 158 (for review, see Mahley and Huang (2006)). While one copy of the ε4 allele is sufficient to increase the risk for AD to two- to threefold, two copies increase the risk to 12-fold (Bertram & Tanzi, 2008).

Importantly the ε4 allele has been genetically linked with a number of other neurodegenerative diseases including tauopathies, Lewy body dementia (Josephs, Tsuboi, Cookson, Watt, & Dickson, 2004), PD (Martinez et al., 2005), and multiple sclerosis (MS; Masterman & Hillert, 2004) and is a well-documented risk factors for AD; T2DM and cardiovascular disease (CVD) (for review, see Hausere et al., 2011). While numerous studies have demonstrated a protective effect for ε2 with respect to AD risk (Corder et al., 1993), the ε2 allele is not entirely benign and has been identified as a risk factor for type 3 hyperlipoproteinaemia and premature atherosclerosis.

ApoE encodes a glycoprotein that is primarily synthesized in the liver and brain. Within the brain, ApoE is mainly secreted by astrocytes and microglia; however, under pathological conditions and selected physiological

conditions neurons also reportedly produce ApoE (Aoki et al., 2003; Xu et al., 1999, 2006). The primary role for ApoE in the periphery and the brain is the maintenance of cholesterol transport and homeostasis (for review, see Hauser et al., 2011). Depletion of cholesterol or deficient cholesterol delivery to neurons results in synaptic and dendritic spine degeneration, decreased synaptic plasticity, and impaired LTP (for review, see Hauser et al., 2011).

There are varying and conflicting hypotheses for the proposed mechanism of increased risk for AD associated with the ε4 allele and protective effect of the ε2 allele. The following section will provide an overview of these hypotheses and associated therapeutic strategies under development (Fig. 2).

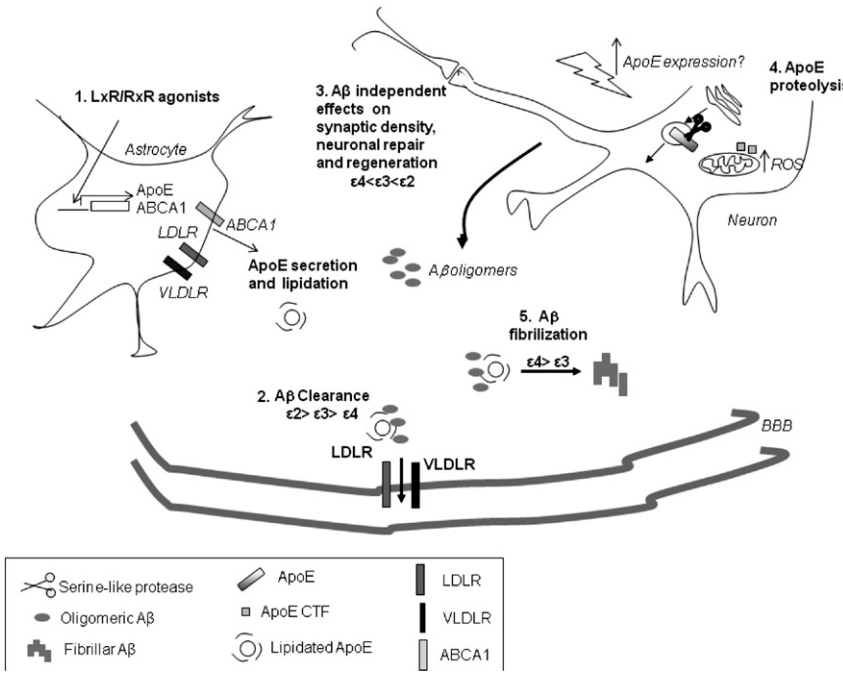

FIGURE 2 Overview of the ApoE-centric therapeutic hypotheses. This figure summarizes the numerous conflicting hypotheses about targeting ApoE-related dysfunction in AD. (1) ApoE secretion and lipidation. LxR and RxR agonists induce expression of ApoE and ABCA1. ABCA1 lipidates ApoE secreted by microglia, resulting in increased affinity for Aβ and increased rate of cellular clearance and across the BBB. (2) ApoE mediated Aβ clearance. The dynamics of ApoE mediated clearance of Aβ are differentially regulated by the different ApoE isoforms, with clearance rates ε2 > ε3 > ε4. (3) Neuroprotection. ApoE exhibits isoform dependent effects on dendritic spine complexity, synaptic density and neuronal repair and regeneration (ε2 > ε3 >ε 4). (4) Neurotoxic role for ApoE ε4. Under conditions of stress, neurons secrete ApoE. The ε4 allele is more susceptible to degradation leading to the formation of toxic C-terminal fragments, which result in mitochondrial dysfunction. (5) Pro-amyloidogenic role for ApoE. The ε4 allele promotes fibrilization of oligomeric Aβ.

1. Targeting Aβ Clearance

ApoE has been demonstrated to have important functions in cellular and BBB clearance of Aβ. Several different transgenic mouse models that express the human ApoE alleles demonstrate an isoform-dependent effect on Aβ pathology, with ε4 expressing mice developing the most severe Aβ pathology (for review, see Tai et al., 2011).

The mechanisms of ApoE-mediated Aβ clearance are dependent on receptor-mediated internalization of ApoE-Aβ complexes, which has been shown to occur through the low-density lipoprotein receptor (LDLR), lipoprotein receptor related protein-1 (LRP1), ApoER2, SorL1, p-glycocprotein, and very low–density lipoprotein receptor (VLDLR) (for review, see Bu 2009). Differential receptor binding between the different Aβ bound isoforms of ApoE have been reported, with ε2 and ε3 complexes preferentially binding to LRP1 and VLDLR and ε4 complexes binding VLDLR (Deane et al., 2008). The dynamics of the rate of clearance with respect to receptor subtype indicates that LRP1 facilitates clearance at a faster rate than VDLR (Deane et al., 2008). A recent publication from the Holtzman laboratory demonstrated that interstitial fluid (ISF) Aβ levels, which closely mirror the patterns of Aβ deposition, were higher in PDAPP/ε4 mice compared to PDAPP/ε2 and PDAPP/ε3 mice (ε4 < ε3 < ε2) and that the rates of clearance from the ISF were significantly longer in ε4 versus ε3 and ε2 (Castellano et al., 2011). Altered clearance of Aβ was demonstrated prior to deposition, indicating that reduced clearance mediated by ε4 isoform directly impacts the level of Aβ deposition (Castellano et al., 2011). An ApoE isoform-dependent affinity for Aβ has also been reported; with ε3 showing two- to threefold greater affinity for Aβ than ε4 (Aleshkov et al., 1997; LaDu et al., 1994; Zhou et al., 1996; Tokuda et al., 2000). Therefore, both decreased ε4 binding affinity for Aβ and decreased rate of clearance appear to contribute to increased amyloid load in ε4 individuals.

Independent of ApoE regulated clearance mechanisms by glia and across the BBB, *in vitro* analysis demonstrated that binding of ε4 to Aβ promotes Aβ fibrilization through altering the conformation of Aβ (Castano et al., 1995; Wisniewski et al., 1994). The Strittmatter laboratory identified $A\beta_{12–28}$ to contain the binding site for ApoE (Strittmatter et al., 1993a; Strittmatter, et al., 1993b). This site has since been the subject of peptide inhibitors that inhibit the ApoE-Aβ interaction, with the hypothesis that this will prevent ε4-induced fibrillization and deposition of Aβ (for review, see Cerf et al., 2011). Such approaches are currently in preclinical development by Wisniewski and colleagues who have demonstrated that inhibiting the binding of Aβ and ApoE with a synthetic nontoxic, non-fibrillogenic peptide of $A\beta_{12–28}$ decreases amyloid burden and cerebral amyloid angiopathy (CAA) and Aβ-induced toxicity in two AD mouse models (Sadowski et al., 2004, 2006). However, blocking this interaction in

the context of the different human ApoE isoforms and potential effects on ApoE clearance mechanisms has yet to be investigated. Immunotherapy approaches to target and inhibit this interaction are also in development, utilizing single domain antibody approaches to enable delivery across the BBB.

2. *ε4 as a Toxic Gain of Function*

Evidence from cell culture, mouse models, and human AD brains demonstrates that ApoE is cleaved by a chymotrypsin-like serine protease generating two populations of C-terminally truncated CTFs; one of 29 KDa and the second ranging from 15 to 20 KDa. It is proposed that the compact structure of the ε4 protein increases its susceptibility to proteolysis (for review, see Mahley et al., 2009).

The 27-KDa fragment has been isolated *in vivo* in the brains of neuron-specific enolase (NSE)-ApoE ε4 expressing mice, peaking at 6–8 months, concomitant with onset of pathology and learning and memory impairments (Brecht et al., 2004). Importantly, accumulation of the 27-KDa fragment did not occur in the GFAP-ApoE Tg model, where ε4 is specifically expressed in glia (Brecht et al., 2004), indicating that these 27-KDa fragments are derived from neuronally secreted ApoE (Brecht et al., 2004); however, this remains to be fully validated. In humans, the 27-KDa fragment has been isolated from cognitively normal individuals of ε3/ε4 and ε4/ε4 genotypes (Brecht et al., 2004;Harris et al., 2003; Huang et al., 2001; Jones et al., 2011). However, these fragments are significantly increased in ε3/ε4 and ε4/ε4 patients clinically diagnosed with AD (Brecht et al., 2004; Harris et al., 2003; Huang et al., 2001). While the full pathological contribution of these CTFs is not clear, evidence suggests that ApoE fragments accumulate in the cytosol, where they have been reported to result in cytoskeletal changes, tau pathology, and mitochondrial dysfunction (Chang et al., 2005; Huang et al., 2001; Nakamura et al., 2009).

Strategies are now under development to target ε4 structure with the aim of restoring normal function and reducing generation of toxic proteolytic fragments. Mahley and colleagues are currently developing small molecules that revert ε4- to ε3-like structure. Ten-day oral administration of the small molecule, PY101 to NSE-A*P*OE ε4 transgenic mice, decreased production of toxic ε4 fragments and increased levels of mitochondrial cyclooxiginase-1 in the hippocampus (for review see Lane et al., 2011). However, whether the decrease in the CTFs produced was sufficient to restore behavioral deficits and neuronal function has yet to be reported. Correcting the structure of ε4- to ε3-like structure would potentially also address Aβ clearance deficits, pro-Aβ aggregation properties of the ε4 allele and restore deficits in cholesterol trafficking and neuronal repair.

3. *ε4 as Loss of Function*

Independent of the effects of ApoE on Aβ deposition and clearance, the ε4 allele clearly has additional effects on synaptic density, dendritic complexity, neuronal repair, and regeneration (for review see Hauser et al., 2011). In contrast to ε3-expressing mice, ε4-expressing mice display synaptic deficits, impaired LTP, decreased numbers of synapses per neuron, and reduced dendritic spine formation (for review, see Hauser et al., 2011).

The hypothesis that ε4 expression levels are lower than that of ε2 or ε3 and that this contributes to the development of AD pathology was supported by mouse studies where an arginine mutation was introduced into murine ApoE to create a human ε4-like mouse ApoE (Ramaswamy et al., 2005; Zhong et al., 2008). Introduction of this mutation resulted in significantly decreased ApoE in the brain, together with synaptic and cognitive deficits (Ramaswamy et al., 2005; Zhong et al., 2008;). Furthermore, targeted replacement of human ApoE isoforms in the PDAPP transgenic mice resulted in expression levels of ApoE, whereby ε4 < ε3 < ε2 in CSF, plasma, and the brain (Bales et al., 2009; Riddell et al., 2008).

Several strategies are now under development to increase ApoE levels. Efforts to identify compounds that increase ApoE and/or ApoE receptor expression levels are underway (Guojun Bu, Mayo Clinic). However, recent studies by the Holtzman group demonstrated that ApoE haploinsufficiency decreased amyloid burden suggesting that increasing ApoE levels without addressing lipidation state may actually increase amyloid burden (Kim et al., 2011). Therefore, further validation of this approach is still required.

4. *ApoE ε2 is Protective*

It is clear that the ε2 isoform is protective in the context of risk for AD, prevents dendritic spine loss in both PDAPP and Tg2576 transgenic mice (Lanz et al., 2003), and increases spine density and connectivity (Dumanis et al., 2011). Efforts are currently underway to deliver the ε2 allele via gene therapy. Steve Paul (Weil Cornell; currently the most clinically advanced program) is developing viral-based delivery methods to deliver the ε2 allele directly into the hippocampus. The group has effectively demonstrated that ε2 delivery into the hippocampus of the PDAPP mouse model, effectively using a lentiviral delivery system, reduced Aβ and maintained expression of ε2 for 12 months (Dodart et al., 2005). However, important preclinical proof-of-concept experiments are yet to be completed to determine if ε2 will be protective in the context of ε4 genotype. If the toxic gain of function of ε4 plays out and the ε4 allele is dominant, introducing expression of ε2 may not confer significant protection. The group is currently completing these important proof-of-concept studies.

Cognosci (http://www.cognosci.com/) is currently developing BBB permeable ApoE peptidomimetics to mimic the effects of the ε3 isoform on

downstream targets including inflammatory pathways and PP2A. Preclinical studies demonstrate a neuroprotective effect in animal models of traumatic brain injury (TBI) (Laskowitz et al., 2010) and stroke (Tukhovskaya et al., 2009). It will be interesting to determine the feasibility of petidomimetics and, again, if this will counteract the potential dominant function of the ε4 allele.

5. *ApoE Lipidation and Function*

The lipidation state of ApoE regulates ApoE binding to Aβ and impacts the clearance of ApoE–Aβ complexes by microglia and across the BBB. Lipidation of ApoE reportedly increases its affinity for Aβ in an isoform specific manner with the binding affinity for Aβ greater for ε2 versus ε3 versus ε4 (Tokuda et al., 2000). ApoE lipidation is regulated by ABCA1, a cellular transporter that mediates cholesterol efflux through binding to ApoE. ApoE and ABCA1 expression levels are regulated by two receptor pairs in the nucleus, PPAR RXR (PPRE) and LXR RXR (LXRE). RXR and LXR agonists have therefore become attractive therapeutic target to increase ABCA1 and ApoE expression and increase ApoE lipidation. Preclinical *in vivo* studies with the RXR activators, GW3965 (Madera Biosciences http://www.maderabiosciences.com/), and Bexarotene (Cramer et al., 2012) and the LXR activator, LT0901317 (Riddell et al., 2007) all demonstrate a reduction in amyloid burden and cognitive improvements (Donkin et al., 2010; Riddell et al., 2007). It is important to note, however, that these studies were performed in transgenic AD models (Madera, APP/PS1; Riddell, Tg2576) that express murine APOE; therefore, the potential effect of this approach in the context of the different human APOE alleles still remains to be tested.

In conclusion, numerous *in vitro* experiments and work in animal models have attempted to elucidate the pathological mechanisms through which the APOE ε4 allele contributes to an increased risk for AD. However, conflicting hypotheses still exist in the field. It is vital that new mechanistic data is generated to determine if ApoE ε4 is a loss or gain-of-function, or a dominant or negative allele. Each of these therapeutic strategies highlighted will provide vital information on proof of mechanism for the pathological contribution of the ε4 allele (For overview see Fig. 2).

E. Synaptic Plasticity and Cognition

AD is ultimately a disorder of synaptic failure leading to cognitive dysfunction. It was first posited in 1975 that the degeneration of dendritic arbors seen in AD may be of clinical significance in addition to neuronal loss (Scheibel et al., 1975). Since then it has been confirmed that synapse loss is indeed a major structural correlate of dementia and likely underlies the cognitive impairments of AD (Terry et al., 1991). Synaptic deterioration

is an early event that occurs well before the formation of amyloid plaques and neuron death (Selkoe, 2002). This degeneration is evident both on an ultrastructural level as abnormal spine morphology and decreased spine densities, as well on a neurochemical level as decreased levels of synaptic proteins. Of all brain regions, the hippocampus is the most severely affected. Even at the MCI stage, there is already substantial synapse loss, which worsens with disease progression. At the mild AD stage, there is a loss of approximately half of the synapses in the CA1 area of the hippocampus (Arendt, 2009).

Soluble oligomers of Aβ have many established detrimental effects at the synapse. Several studies have demonstrated that Aβ oligomers preferentially bind to, or cluster, at synapses, resulting in changes in spine morphology and decreases in spine density (Selkoe, 2008). Disrupting this interaction has therapeutic potential for preserving neuronal function. However, the identity of the Aβ-binding partner(s) that mediate such effects remains unclear and controversial.

All currently available FDA-approved drugs for AD involve modulation of neurotransmission. Donepezil (Aricept; Eisai, Pfizer), galantamine (Razadyne; Ortho-McNeil-Janssen), rivastigmine (Exelon; Novartis), and tacrine (Cognex; Sciele Pharma) are all inhibitors of acetylcholinesterase (AChE), except Memantine—an NMDA antagonist. Unfortunately, data from the nearly two decades of experience with these drugs indicate that efficacy is limited, with only mild improvements in cognition and without lasting effect on disease progression (Hansen, Gartlehner, Lohr, & Kaufer, 2007). In addition, only 25–50% of AD patients respond to the treatment with these drugs (Giacobini, 2000).

Given the minimal efficacy of current AD drugs, scientists are developing a variety of novel nootropic therapeutic strategies that target synaptic plasticity and cognition, with multiple compounds in clinical testing. Most of these strategies focus on various neurotransmitter systems, although there are also novel targets that could lead to cognition enhancement via nontransmitter mechanisms.

1. Cholinergic

Besides raising overall levels of ACh by preventing its degradation, targeting the receptors themselves is another option for improving cognition. Muscarinic receptors are G-protein-coupled metabotropic ACh receptors that are widely distributed throughout the peripheral and CNS. In the CNS, muscarinic receptors can be located pre- or postsynaptically and have been shown to be involved in memory and attention processes. Overall, compounds targeting this receptor class have been difficult to develop due to lack of selectivity, toxicity, and significant cholinergic-mediated side effects (Mangialasche et al., 2010). However, allosteric modulators of muscarinic receptors offer an alternative approach that may avoid some of the

selectivity and side effect issues of agonists. Preclinical data indicate that one such compound, benzyl quinolone carboxylic acid (BQCA), can selectively potentiate M1 receptors and improve cognitive symptoms in animal models (Shirey et al., 2009).

Nicotinic acetylcholine receptors (nAChRs) offer additional cholinergic targets for enhancing cognition. These ionotropic receptors are distributed throughout the brain in both the postsynaptic compartment, where they participate in excitatory neurotransmission, and the presynaptic compartment, where they function in modulating neurotransmitter release. Nicotinic receptors have been heavily studied for their role in memory and attention processes. Numerous studies have demonstrated that agonists and partial agonists have the ability to improve cognitive performance in animal models and humans (Gotti et al., 2006). Various compounds from this category have been tested in clinical trials for AD. RO5313534 (Roche) is a selective partial agonist for the α7 nAChR subtype and additionally has 5HT3 receptor antagonist properties. This compound is currently in clinical development as an add-on for approved AD drugs such as donepezil and memantine. EVP-6124 (EnVivo) is another potent α7 nAChR agonist that is currently in Phase II clinical testing for AD. The other major neuronal nAChR subtype, α4β2, is also targeted for potential cognition-enhancement effects. Agonist compounds such as AZD1446, AZD3480 (AstraZeneca, Targacept), ABT-089 (Abbott), and varenicline (Chantix; Pfizer) are in clinical development. A recent phase I clinical trial also demonstrated the potential of using nicotine as a direct agonist to improve cognition. A transdermal nicotine patch, which is currently used as a smoking cessation treatment, was found to improve cognitive measures in patients with MCI (Newhouse et al., 2012).

Another approach is to increase the number of AChRs present at the plasma membrane. VILIP-1 is a neuronal calcium sensor protein that has been shown to participate in the exocytosis and surface expression of α4β2 nAChRs. Furthermore, VILIP is down-regulated in multiple regions of the brain in AD (Zhao et al., 2009). Therefore, modulating VILIP expression or activity is a potential therapeutic strategy for increasing AChR expression and is currently in preclinical development.

2. *Monoaminergic*

In addition to cholinergic neurotransmission, monoaminergic systems also become dysfunctional and undergo neurodegeneration in AD. In particular, the serotonergic neurons of the raphe nuclei and the noradrenergic neurons of the locus coeruleus exhibit early and progressive degeneration (Zweig et al., 1988). Since monoaminergic signaling can modulate the other neurotransmitter systems, dysfunction can negatively impact cognition via many pathways including regulation of ACh release. To address

the serotonergic dysfunction in AD, the 5-HT1A, 5-HT4, and 5-HT6 receptors have been targeted. Xaliproden (SR57746; Sanofi-Aventis) is a 5-HT1A agonist, which was tested in Phase III trials for mild to moderate AD, with unsuccessful results. PRX-03140 (Epix), a partial 5-HT4 receptor agonist, is proposed to have multiple beneficial effects including increasing ACh release and neurotrophic factors and decreasing Aβ levels. 5-HT6 receptor antagonist strategies have also demonstrated positive cognition-enhancing effects for multiple neurological disorders. SB-742457 (GlaxoSmithKline) is a selective 5-HT6 antagonist that has demonstrated preliminary efficacy in Phase II trials for AD. Other 5-HT6 antagonist compounds are also in clinical development, including SYN-114 and SYN-120 (Synosia, Roche).

Dopaminergic signaling has also been targeted in AD drug discovery. R-pramipexole is a dopamine receptor agonist that is currently approved for treating early-stage PD. This drug displays agonist activity for the D2S, D2L, D3, and D4 dopamine receptors and may also have additional antioxidant and neuroprotective properties (Piercey, 1998). This drug is planned to go a Phase II trial for early AD. An alternative approach to increasing dopamine levels is to inhibit monoamine oxidase B (MAO-B), an enzyme that breaks down dopamine. EVT-302 (Evotec, Roche) is a potent and select MAO-B inhibitor that will also enter Phase II clinical trials.

Another monoamine neurotransmitter that has potential for AD therapeutics is histamine. Histamine receptors of the H_3 subtype are expressed throughout the CNS in multiple neuron types. One of the functions of H3 receptors is presynaptic regulation of the release of other neurotransmitters, including ACh, dopamine, serotonin, and GABA. Activation of H3 receptors, which are G-protein coupled, leads to modulation of calcium channels; this in turn results in reduced calcium influx into the presynaptic terminal, limiting the action potential response and neurotransmitter exocytosis (Bonaventure et al., 2007). Therefore, inhibiting H3 receptors in order to increase transmitter release could have a significant impact on cognition. SAR110894D (Sanofi-Aventis) is one such antagonist compound that is currently in Phase II clinical trials for mild to moderate AD.

Adrenergic neurotransmission has also been targeted in AD drug discovery. Epinephrine and norepinephrine are both hormones and neurotransmitters with multiple roles throughout the body and CNS. Norepinephrine reuptake inhibitors (NRIs) are a class of drugs that blocks the norepinephrine transporter, thereby raising levels of this transmitter. Recently, a drug in this class that is already approved for attention deficit hyperactivity disorder, atomoxetine (Strattera; Eli Lilly), was tested in a Phase II/III as an augmentation treatment for AD patients already taking AChE inhibitor drugs. This study did not find any significant improvement in cognition when this drug was added to cholinesterase inhibitor therapy (Mohs et al., 2009), although further testing is continuing.

3. Excitatory Neurotransmission

Recent data indicate that metabotropic glutamate receptors are also involved in Aβ pathology and therefore may be possible therapeutic targets for drug development. In particular, the mGluR5 receptor subtype appears to interact with Aβ oligomers and precipitate synaptic pathophysiology (Renner et al., 2010; see Section II.A). Currently, mGluR antagonists and allosteric modulators are in clinical development for other neurological indications, including PD, schizophrenia and depression.

An alternative approach to enhancing glutamatergic neurotransmission is to increase the number of receptors at the synapse. One of the effects of Aβ oligomers is the dysregulated internalization of glutamate receptors, including both AMPA and NMDA receptors. Since dephosphorylation is a key regulatory mechanism in this process, blocking relevant phosphatases is a possibility for preventing this type of synaptic deficit. Striatal-enriched phosphatase (STEP) is a tyrosine phosphatase that is involved in the internalization of glutamate receptors and has been implicated in a variety of neurological disorders including AD. Reducing STEP levels was shown to prevent Aβ-induced receptor internalization, as well as to rescue cognitive deficits in an AD mouse model (Fitzpatrick & Lombroso, 2011). Based on this evidence, development of STEP inhibitor compounds is a potential therapeutic strategy.

Along the lines of the anti-excitotoxic approach of memantine, increasing inhibitory tone with non-GABAergic anticonvulsant drugs may also avert excitotoxic neuronal damage. An example of this approach is levetiracetam (Keppra; UCB), an anti-seizure medication used to treat epilepsy. Levetiracetam has multiple mechanisms of action that enable modulation of neuronal activity, including inhibition of AMPA receptors and intracellular calcium elevations as well as binding to synaptic vesicle protein 2A (SV2A; Surges et al., 2008). A Phase II clinical trial in MCI patients is in progress; preliminary data indicate that levetiracetam treatment is successful in decreasing hippocampal hyperexcitability and improving cognition.

4. Calcium Channels

A large amount of research suggests that disruption of calcium homeostasis plays an important role in AD. In particular, dysregulated calcium influx may lead to neuronal dysfunction and eventually cell death. (Bezprozvanny & Mattson, 2008). Using this as a rationale, neuronal calcium channels have been targeted for drug development. An example compound is nimodipine (Nimotop; Bayer), a blocker of L-type voltage-gated calcium channels that was originally developed as an antihypertensive drug. In several European countries, it is also frequently prescribed for cognitive impairment and dementia. However, it failed to gain FDA approval in the

US due to unconvincing data from multiple clinical trials, which demonstrated limited cognitive improvement but no long-term benefit (Lopez-Arrieta & Birks, 2002).

5. *Phosphodiesterase*

The secondary signaling molecules, cGMP and cAMP, are critical participants in many cellular signaling pathways, including those involved in learning and memory. As such, targeting the metabolism of these molecules has been an actively pursued therapeutic approach for AD. The cyclic nucleotide phosphodiesterases (PDEs) are enzymes that break down cAMP and cGMP. There are 11 families of PDEs, although for cognition-enhancement research, the primary isoforms targeted include PDE2, PDE3, PDE4, PDE5, PDE9 and PDE10 (Reneerkens et al., 2009).

Example compounds in this class include cilostazol (Pletal; Otsuka), a PDE3 inhibitor that increases cAMP levels and is approved for the treatment of intermittent claudication. It is currently being tested in a phase IV clinical trial in South Korea as an adjunctive therapy for AD patients taking donepezil. Other PDE inhibitors tested in clinical trials include MK0952 (Merck), a PDE4 inhibitor that completed a Phase II trial in 2007, and EHT 0202 (ExonHit Therapeutics), a multi-targeted PDE4 inhibitor that completed a Phase IIa trial in 2009. EHT 0202 is also a modulator of GABA receptors and the α-secretase APP pathway and therefore could exert beneficial clinical effects via multiple mechanisms (Vellas et al., 2011).

PDE5 is another promising target PDE family, one for which there are approved inhibitors for the treatment of erectile dysfunction (e.g., sildenafil, tadalafil, vardenafil). Sildenafil (Viagra; Pfizer) has been demonstrated to improve memory and synaptic plasticity measures in a mouse model of AD (Puzzo et al., 2009). However, the existing marketed PDE5 inhibitors are not ideal for treating AD due to issues in isoform selectivity and BBB penetrance, therefore developing improved PDE5 inhibitors is an active area of preclinical development.

6. *Epigenetic Modulators*

Several recent studies have demonstrated the involvement of epigenetic mechanisms in normal learning and memory, as well as multiple neurological pathologies including AD (Day & Sweatt, 2011). In particular, histone acetylation appears to become dysregulated with both age-dependent memory impairment and amyloid-induced pathology in mice. Treatment with histone deacetylase (HDAC) inhibitors is able to rescue deficits in memory and synaptic plasticity in the animal models (Peleg et al., 2010). There are two approved HDAC inhibitors and many in clinical testing in the cancer field. For AD, one clinical candidate is nicotinamide, the amide of vitamin B3

(niacin) and an inhibitor of the sirtuin HDAC family. In transgenic AD model mice, treatment with nicotinamide rescued cognitive dysfunction (Green et al., 2008). It is currently in Phase II clinical trials for mild to moderate AD. Other HDAC inhibitors are in preclinical development, including ones specific for certain HDAC isoforms. Histone acetyltransferases, which are the acetylating counterparts to HDACs, are another potential target for cognition-enhancement.

Prior to neuron death, in the asymptomatic preclinical stages, synaptic abnormalities are likely reversible events that can be targeted pharmacologically. This further emphasizes the need for early diagnosis and intervention, when AD pathology is the most amendable to modification. Although current neurotransmitter-based drugs are limited to symptomatic treatments, the possibility that a synaptic plasticity-targeted compound could be disease-modifying is not excluded since synaptic activity is crucial for neuronal viability. Depending on what future research uncovers about AD etiology, hitting the right synaptic targets may have significant downstream effects in preventing neurodegeneration. In addition, even with the discovery of disease-modifying treatments, drugs that acutely target synaptic plasticity would still be necessary for treating symptomatic AD since the effects of disease-modifying compounds on cognition may not be immediate. A combination therapy in such cases would be ideal, involving both acute cognition enhancement/maintenance as well as chronic modification of disease pathways (see Table III for overview of current clinical trials).

TABLE III Overview of the Current Synaptic Targeted Programs in Clinical Trials

	Synaptic Targets		
Drug Name	*Target*	*Investigator*	*Phase*
Single-target			
R05313534	α7 nAChR	Roche	II
EVP-6124	α7 nAChR	EnVivo	II
AZD3480	α4β2 nAChR	Targacept	II
Varenicline	α4β2 nAChR	Pfizer	II
SB-742457	5-HT6	GSK	II
SYN-114, SYN-120	5-HT6	Roche	I
R-pramipexole	Dopamine	VCU, ADDF	II
EVT-302	Monoamine oxidase B	Roche	II
SAR110894D	H3 histamine	Sanofi-Aventis	II
Cliostazol	Phosphodiesterase 3	Seoul National Hospital	IV
EHT0202	Phosphodiesterase 4	Exonhit	II
Nicotinamide	HDAC	UCL, AA	II
Multi-target			
Ladostigil	AChE, BChE, MAOA/B	Avraham	II
Levetiracetam	AMPAR, SV2A	JHU	II

F. Neurotrophic/Neuroprotective Strategies

1. Neurogenesis

While current AD therapies do offer modest symptomatic relief, none address the major underlying biology of AD related to synaptic alterations and cell loss that leads to memory processes. The discovery of *de novo* production of neurons in the adult dentate gyrus has introduced the possibility of a novel form of plasticity that might sustain memory processes (reviewed in Mu & Gage, 2011). This growing body of evidence supports the hypothesis that promotion of adult hippocampal neurogenesis might improve pattern separation and spatial memory in the clinically normal population (Sahay et al., 2011). In contrast, reduced hippocampal neurogenesis has been associated with aging, and appears to be hypoactive in disorders such as AD (Clelland et al., 2009). Accumulation of Aβ in AD (Muresan et al., 2009) has been associated with impaired adult neurogenesis, as well as impairments in autophagic/lysosomal clearance (Koga et al., 2011).

Understanding the molecular mechanisms associated with alterations in neurogenesis observed at early and later stages of AD, and in association with normal aging, will contribute to the development of novel AD biomarkers, reveal insight into the pathogenesis of AD, and will provide novel insight toward therapeutic targets to promote repair of AD-related lesions. Regulators of hippocampal neurogenesis offer the potential to repair and/or rebuild lost neural circuits, which result from cell death associated with the aberrant accumulation of intraneuronal NFTs and intra- and extra-neuronal toxic accumulation of Aβ. A proneurogenic therapeutic strategy for AD presents a particularly alluring prospect of a class of late-stage intervention and/or reversal therapies, based on the theory that proneurogenic compounds might promote the repair of lost circuits via the *de novo* replacement of dead cells. In this section, we will discuss some of the relevant biology and considerations for developing neurogenesis-related therapeutics.

Seminal studies demonstrated that neurogenesis in adult mammals occurs in two regions of the brain: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (Matsuzaki et al., 2004). This process involves the proliferation of neuronal precursor cells and their subsequent maturation is important for regular maintenance of the normal structure and function of the hippocampus, and is a requisite for hippocampus-dependent learning and memory (reviewed in Lee et al., 2011). Remarkably, neurogenesis in the dentate gyrus serves as a source of neurons that integrate locally into the granular layer of the dentate gyrus, displaying lifelong structural and functional plasticity. During neurogenesis, the majority of newly formed hippocampal neural precursors undergo apoptosis prior to maturation, whereas the surviving precursors mature to

become fully incorporated and functional neurons (for review, see Mu and Gage, 2011).

Several experimental paradigms have been associated with enhanced adult hippocampal neurogenesis in mice, including studies of environmental enrichment (Hu, Xu, et al., 2010) and voluntary exercise (van Praag et al., 1999). Fibroblast growth factor-2 (FGF-2), some antidepressants (Schmidt & Duman, 2007), allopregnanolone (Wang, Singh, et al., 2010), and a novel class of small molecules (including the recently identified P7C3; Pieper et al., 2010) are also known to enhance neurogenesis in adult mice and/or humans. Among many genes reported to impact adult neurogenesis is the gene encoding NPAS3, a CNS-specific transcription factor that is associated with mental illness and learning deficits (Macintyre et al., 2010). *Npas3* knockout mice display a profound loss of hippocampal neurogenesis (Pieper et al., 2005), atypical dentate granular cell morphologies and alterations in synaptic transmission (Pieper et al., 2010), as well as behavioral abnormalities (Erbel-Sieler et al., 2004). The small molecule P7C3 was discovered through an *in vivo* screen for molecules capable of restoring hippocampal neurogenesis in *npas3* knockout mice, and was shown to exhibit remarkable anti-apoptosis activity (Pieper et al., 2010). Prolonged administration of P7C3 to aged rats enhanced neurogenesis in the dentate gyrus, impeded neuron death, and preserved cognitive capacity as a function of terminal aging (Pieper et al., 2010), lending support for the development of small molecule regulators of neurogenesis as potential AD therapeutics.

2. *Neurotrophins*

Converging lines of evidence on the possible connection between neurotrophin signaling and AD are stimulating new therapeutic directions focused on NGF and BDNF. Neurotrophins are secreted peptides that act on cell surface receptor to promote the differentiation, growth, and maintenance of developing neurons, the survival of adult mature neurons, and regulate synaptic plasticity (for review, see Calissano et al., 2009). Neurotrophins, including the prototypical NGF, have been studied extensively for their ability to prevent neuronal atrophy that is observed in AD.

Mature NGF levels are reduced in the basal forebrain of AD patients, resulting in cellular shrinkage, downregulation of transmitter-associated enzymes (i.e., choline acetyltransferase and acetylcholinesterase), and reduction in nerve fiber density (for review, see Calissano et al., 2009). In contrast to these changes, levels of proNGF are increased in the frontal and occipital cortex and in the hippocampus at late-stage AD, as well as in patients with MCI (Peng et al., 2004). While this may suggest a role of impaired conversion to mature NGF from its pro-form, it is worthwhile to note that the survival/death choice of NGF-dependent neurons depends on an intricate balance between mature NGF and pro-NGF (Lu et al., 2005), and the spatial and temporal expression of the distinct NGF receptors (TrkA, p75, and

Sortilin; for review, see Calissano et al., 2009). These findings suggest that aberrant processing of proNGF and/or altered axonal transport of NGF may contribute to the onset of AD-related neurodegeneration. Compelling *in vivo* evidence in the AD11 mouse line (expressing recombinant anti-NGF antibodies) indicates a 50% reduction in endogenous NGF is sufficient to mimic the sporadic AD phenotype, including loss of basal forebrain cholinergic neurons, accumulation of hyperphosphorylated tau, accumulation of Aβ plaques, and deficits in synaptic plasticity (Cattaneo et al., 2008; Origlia et al., 2006).

Taken together, these studies indicate targeting NGF signaling as a potential therapeutic avenue for AD. Indeed, several therapeutics targeting NGF are under study. Intranasal administration of NGF in combination with oral administration of ganstigmine and dopenazil in the AD11 mouse improved performance in hippocampus-dependent spatial memory tasks (Origlia et al., 2006). Implantation of genetically modified autologous fibroblasts (to express human NGF) into the forebrain of mild AD patients is currently in Phase I clinical trials and has been associated with a reduction in rate of cognitive decline, and no adverse events have been associated with up to 24 months of *ex vivo* NGF gene delivery (Tuszynski et al., 2005). Other strategies involve targeting small molecule ligands of the neurotrophin receptor p75. These may act to block Aβ-induced activation of calpain/CDK5, GSK-3β, c-Jun and tau phosphorylation, as well as act to prevent Aβ-dependent AKT and CREB inactivation. In animal studies these small molecules have been demonstrated to inhibit Aβ-induced neuronal death (Yang et al., 2008; also see Longo "Neurotrophin receptor activators for the treatment of Alzheimer's disease"). In other studies, differentiated PC12 cells that were deprived of NGF accumulated intra- and extra-cellular Aβ immediately preceding the onset of apoptotic death, which could be abrogated with β-or γ-secretase inhibitors (Matrone et al., 2008). Since there is an intimate relationship between NGF signaling and amyloidogenesis, NGF-regulating strategies may represent a more specific avenue for promoting cell viability, while indirectly modulating the accumulation of toxic protein aggregates.

Apart from the canonical effects on neuronal differentiation, neuritic outgrowth, and survival, neurotrophins also play a fundamental role in synaptic transmission and plasticity (for review, see Alberini, 2011). Recently, insulin-like growth factor II (IGF-II), a mitogenic polypeptide that is important in normal somatic growth and development, tissue repair, and regeneration, was found to be highly concentrated in the hippocampus and to be regulated as a C/EBP target gene with a functional role in memory formation (Chen et al., 2011). IGF-II-dependent memory enhancement requires IGF-II receptors, new protein synthesis, and correlates with a significant activation of synaptic GSK-3β and increased expression of GluR1 (Chen et al., 2011). While any role of aberrant IGF-II signaling in AD remains to

be determined, long-term memory modulating effects of IGF-II present a novel avenue for the development of pro-cognitive therapies, which may have high value in the AD clinical population.

G. Inflammation

1. Biology

Early interest in the role of immunological mechanisms in brain aging and AD began with the "modern" era of Alzheimer's research in the 1970s. Nandy was among the first to suggest that immune mechanisms were involved, describing "anti-brain" antibodies using immunohistochemical techniques, demonstrating there were "anti-neuronal" antibodies in the sera of aged mice and nonhuman primates, which correlated with impaired learning. Fillit et al. (Bradford et al., 1989; Fillit et al., 1987; Foley et al., 1988) studied the specificities of anti-brain antibodies. Anti-neuronal antibodies were demonstrated to have cholinergic neuronal specificity, and these human auto-antibodies from patients with AD were shown to be functional, where they were cytotoxic to cholinergic neurons *in vitro* (Bradford et al., 1989; Foley et al., 1988). Anti-vascular autoantibodies were also found to play a role in BBB impairment in AD (Fillit et al., 1987). Subsequent studies by Michaelson and others also demonstrated the presence of autoantibodies to neurofilament proteins, Aβ in sera of patients with AD; however, titers of such antibodies have not proven diagnostic in all clinical studies (Nath et al., 2003).

In general, immune mechanisms play an important role in aging (Vallejo, 2011). While humoral immunity declines with aging (Frasca & Blomberg, 2011), cellular immunity and inflammatory responses tend to remain constant through the life span. With aging, immune clearance via naturally occurring autoantibodies is less efficient in removing damaged tissue, and antibodies are also less effective in all forms of immune reactions, including antiviral and antibacterial reactions. A general increase in tissue damage and degradation may result in the increase in tissue specific antibodies, and the entire system may become generally overwhelmed. Auto-antibodies specifically recognizing misfolded proteins, or proteins modified by oxidation or other damaging reactions, resulting in "altered" native antigens being recognized as foreign, are common (e.g., see Moir et al. (2005); Perez-Garmendia et al. (2010)). In this context, a failure of clearance is not necessarily a primary driver, but rather secondary to tissue damage resulting from a variety of causes such as oxidation. Applying these concepts to AD, one would predict that the decline in immune clearance would lead to the accumulation of damaged tissue in brain, such as Aβ, although other immunologic mechanisms may also play a role in AD (Bouras et al., 2005).

2. *Immunotherapy*

These findings contributed to the concept in current clinical trials of immunotherapy for AD employing monoclonal antibodies as "passive" vaccines, with some interesting initial findings in Phase II studies (Bohrmann et al., 2011; Panza et al., 2011; Samadi & Sultzer, 2011). In addition, the finding of naturally occurring autoantibodies to Aβ (and several other self antigens in the sera of elderly patients and controls) prompted the idea of employing IVIg as a therapy for AD (for review, see Balakrishnan et al., 2010; Fillit, 2004), currently in Phase III. As expected, antibodies to Aβ are present in IVIg. Current clinical immunotherapy programs employing monoclonal antibodies target different epitopes spanning the entire domain of Aβ, as well as monoclonal antibodies presumably reacting with Aβ oligomers and aggregates. Each approach has its advantages and proponents. Some argue that anti-Aβ N-terminal antibodies are more effective because this domain is accessible in plaque deposits to antibody, and is not buried and hidden within aggregates. Other variations on monoclonal antibodies include the use of Fc receptor approaches, with the concept that recruitment of microglial to the plaques is critical in enhancing clearance (Bacskai et al., 2002). Presumably, Fc receptor activity is not critical to a mechanism of action in which monoclonal vaccines are preventing oligomer or fibril formation, or binding to Aβ in the plasma through a "sink" effect. IgG subtype may also be important in determining variation in BBB penetration and efficiency of complement binding.

More recently, immunotherapy has been directed toward tau, employing monoclonal antibodies targeting tau-related epitopes, including monomers and oligomers (Sigurdsson, 2009). While effective in animal models, these approaches have not yet been tried in humans. Finally, "active" vaccines are also currently still in development despite early indications of a safety signal. An active vaccine would have practical value with respect to requiring injections on a limited scale, instead of requiring lifelong infusions. Active vaccines seek to induce a humoral response, without a T cell response, through immunologic engineering of the vaccine itself (Lemere, 2009). However, long-term safety remains a serious concern, as initial studies were associated with serious side effects (Nitsch & Hock, 2008).

3. *Anti-Inflammatory*

In the early 1980s, immunopathological studies began to demonstrate signs of inflammatory reactions surrounding and within the senile plaques (for review, see McGeer & McGeer, 2003). Most of the components of both a cellular and a humoral immune response were through immunohistochemical studies of autopsied brain from patients, including immunoglobulin, complement components, and infiltrating microglia. While the inflammation seen in autopsy studies of AD is not nearly as robust as that seen in

more typical inflammatory autoimmune disease, such as MS, inflammation has been a target for new drug therapy in AD for many years and is generally viewed as a contributing factor to disease pathology and progression (Gorelick, 2010). The human pathology suggesting inflammation in AD has been supported by much epidemiology, suggesting that the use of anti-inflammatory agents was associated with a reduction in the risk of AD (McGeer & McGeer, 2003). The first clinical trial to test the immune hypothesis was the use of prednisone, a broadly effective anti-inflammatory with good brain penetration. While there were surprisingly few adverse reactions in this relatively brief trial, no efficacy was noted (Aisen, 2000).

Epidemiological studies suggest associations between the use of anti-inflammatory agents, primarily nonsteroidal anti-inflammatory agents (NSAIDs), and a reduced risk of AD. Some data indicated that specific classes of NSAID agents might be particularly associated with efficacy, including aspirin. This lead to several large clinical trials in both academia and industry of several NSAIDs, all of which failed (Trepanier & Milgram, 2010).

Newer approaches to inhibiting inflammation in the Alzheimer's brain remain of interest. Cytokines have long been thought to play an important role in AD, causing neuronal cytotoxicity, and increasing Aβ production, as well as other extracellular matrix components that compose the reparative response *in vitro* (Garcia de Yebenes et al., 1999; Leveugle et al., 1995; Mattson et al., 1997). Signs of inflammation are a characteristic feature of aging and AD. Increased blood markers of inflammation including (Buchhave et al., 2010) cytokines such as IL-1, IL-6, and TNFα, have been described (Angelopoulos et al., 2008; Fillit et al., 1991; Jiang et al., 2011; Swardfager et al., 2010). In addition, elevated C-reactive protein (CRP), considered a biomarker of the systemic inflammatory response, was elevated in some, but not all, AD studies (Bettcher et al., 2012; Mancinella et al., 2009; Roberts et al., 2009). However, these studies must be considered carefully from a clinical design perspective, since patients with AD often suffer infections, are generally more frail, and suffer more medical comorbidities, all of which may cause systemic inflammation (Cunningham, 2011). Some have even postulated that recurrent infections in the elderly, as well as increasing tissue damage with aging resulting in an inflammatory reaction, could cause increased levels of cytokines, ultimately affecting the brain and resulting in, or contributing to, the initiation and progression of neuronal injury and/or Aβ deposition—characteristics hallmarks of AD (Holmes et al., 2009). These studies of cytokines suggest novel therapeutic approaches, such as TNFα blockers (including thalidomide (Greig et al., 2004) and Enbrel (Clark et al., 2010; Tweedie et al., 2007).

Others have approached the issue of inflammation from the perspective of the microglia (Cameron & Landreth, 2010), and some have postulated that blocking microglial function as an anti-inflammatory approach might

have therapeutic benefit in reducing cytotoxicity, such as blockade of specific microglial kinases (Watterson et al., 2001). Others have speculated that impaired microglial function might contribute to Aβ accumulation and persistence in the brain, and have attempted to increase microglial function through interventions such as G-CSF or GM-CSF (Boyd et al., 2010; Sanchez-Ramos et al., 2008, Sanchez-Ramos et al., 2009), and clinical trials with this approach are currently underway.

Finally, immunologic studies have contributed to the development of novel biomarkers for AD. Most CSF markers require specific and avid monoclonal antibodies for detection of Aβ and various forms of tau. Perhaps more directly, while various autoantibodies in plasma have failed to have biomarker significance, recent studies have suggested that antibody "signatures" might have diagnostic value (Nagele, Clifford, et al., 2011; Nagele, Han, et al., 2011; Restrepo et al., 2011). Along these lines, recent studies have suggested that TNFα, as a marker of systemic inflammation, has considerable value in predicting the rate of change in cognition in AD patients and should be considered a confounding variable in estimating the rate of change in disease modifying clinical trials (Diniz et al., 2010; Holmes et al., 2009) Principles of the "immunology of aging" are relevant to AD pathogenesis and treatment. As degenerative changes occur, the immune system plays a critical role in monitoring for damaged tissue and removing through both cellular and humoral immune responses. These reparative mechanisms may decline with age, leading to the accumulation of damaged, misfolded, or oxidated proteins as a result of a failure of clearance. Furthermore, as immune mechanisms become dysregulated with age, autoimmune and inflammatory processes may ensue, which can further damage tissue via cellular cytotoxicity, including neurons. As a result, inflammation can become independently activated. All of these mechanisms appear to be at work in AD, and all represent potential therapeutic targets for intervention. While numerous clinical trials have tested anti-inflammatory drugs to date and all have failed, the first immunotherapy to be carefully tested in Phase III trials, the monoclonal antibodies to Aβ, should report their findings in the coming year, and will certainly be most interesting.

H. Metabolic Dysfunction in Alzheimer's Disease

The brain exclusively uses glucose for energy and consumes 25% of the body's glucose supply. Therefore, the brain is particularly susceptible to metabolic dysfunction. One of the earliest pathological events in AD is brain hypometabolism and reduction in glucose utilization, as detected by fluorodeoxyglucose (FDG)-PET neuroimaging. Studies suggest that FDG-PET signal decreases decades before AD diagnosis and correlates very tightly with clinical symptoms and disease progression (Mosconi et al., 2008). Scientists in the field have debated whether this decrease in energy

utilization and neuronal function is a cause or a consequence of dying neurons. Although given the early onset of bioenergetic dysfunction in the disease process, there is hope that intervening in this process would alter the disease trajectory, boost neuronal function, and prevent cell death.

Epidemiological studies have shown an association between type 2 diabetes and AD (Ott et al., 1996). Twenty-three percent of patients over the age of 65 with dementia are also reported to have diabetes (Alz.org, 2011, Facts and Figures). Diabetes is also more common in those with MCI and impaired acute insulin response at midlife is associated with risk for AD in later life. Animal studies have also shown a correlation between insulin resistance and AD. AD mice fed a high-fat diet (Li et al., 2003) or fed sucrose-sweetened water (Cao et al., 2007) to develop enhanced Aβ pathology. In both of these paradigms, mice develop insulin resistance. Further, caloric restriction, which increases insulin sensitivity, attenuates Aβ pathology in mice (Patel et al., 2005). Several transgenic mouse models also demonstrate increased pathology when both diseases coexist. In two separate studies that generated AD transgenic mice with a diabetic phenotype ([APP23]×[ob/ob], [APP23] × [NSY], APP/PS1 with STZ-induced insulin deficiency), the diabetic phenotype promoted Aβ deposition and deficits in spatial memory, while the presence of the human APP transgene exacerbated the diabetic phenotype (Takeda et al., 2010; Wang, Zheng, et al., 2010).

A genetic link between type 2 diabetes and AD has also been recently described where GWAS studies identified *SorCS1* as a genetic risk factor for both type 2 diabetes (Goodarzi et al., 2007) and late-onset AD (Liang et al., 2009). Characterization of *SorCS1*-deficient mice revealed insulin resistance (Pedersen et al., 2010), which was paralleled by increased brain Aβ accumulation (Lane et al., 2010). Gandy and colleagues subsequently highlighted a role for SorCS1 in the intracellular sorting of APP and regulation of Aβ generation (see Section II.C; Lane et al., 2010).

It is clear that diabetes and AD share common disease mechanisms. Excessive caloric intake and diabetes are associated with damage to vital brain regions that are relevant to learning and memory, as well as to brain microvascular function. High levels of insulin and triglycerides in the blood are associated with an inflammatory response in the brain (Fishel et al., 2005). Type 2 diabetes can also result in increased oxidative stress, which may also increase risk for AD. Excess calories require excess metabolism, resulting in the production of damaging free radicals. The microvascular dysfunction associated with diabetes can also lead to micro-hypoxic events, damaged mitochondria, and the promotion of oxidative stress and impaired neuronal function. Mitochondria generate the majority of the cell's ATP and are also involved in signaling, cell growth and differentiation, and cell death. Given the vital functions of the mitochondria, it is no surprise that mitochondrial dysfunction plays an important role in diabetes, as well as

many neurodegenerative diseases, highlighting the sensitivity of brain cells to proper bioenergetic functioning. There are also studies directly implicating insulin receptor signaling in APP biology. Stimulation of insulin/IGF-1 receptor by ligand binding results in initiation of downstream signaling cascades including activation of phosphotidylinositol-3-kinase (PI3K; Taniguchi et al., 2006). Activation of PI3K has been implicated in downstream events of insulin/IGF-1 signaling such as survival, cell growth, and trafficking of the glucose transporter 4 (GLUT4) to the plasma membrane from storage vesicles to facilitate glucose uptake (Bai et al., 2007). Stimulation of cells with insulin increases Aβ and sAPP secretion in a PI3K dependent manner (Adlerz et al. 2007; Gasparini et al. 2001; Solano et al. 2000). Further, PI3K inhibition retards Aβ and sAPP secretion (Gandy, 1999; Solano et al., 2000).

Insulin also influences hyperphosphorylation of tau (Hong et al., 1997; Hong & Lee, 1997). Insulin signaling can inhibit GSK-3, a protein previously shown to affect both NFT and senile plaque pathology in AD. Diabetic mice with reduced insulin signaling and presumably higher GSK-3 activity show increased tau phosphorylation (Planel et al., 2007). GSK-3 was shown to phosphorylate tau protein in human neuronal cells (Hong & Lee, 1997) and studies have also shown that increased GSK-3 activity may regulate Aβ generation by increasing γ-secretase activity (Phiel et al., 2003). Therefore, insulin dysregulation could contribute to AD pathology by affecting both amyloid and tau pathogenic pathways in addition to pathways associated with general aging.

Another potential shared molecular pathway between diabetes and AD centers on the insulin degrading enzyme (IDE). IDE is responsible for degrading insulin and can also degrade Aβ (Craft & Watson, 2004). High levels of insulin reduce Aβ degradation, reflecting in increased CSF Aβ in a case-control study (Craft & Watson, 2004). In type 2 diabetes, insulin resistance leads to increased circulating levels of insulin, possibly sequestering the available IDE and limiting Aβ degradation. Higher levels of Aβ are then available to fuel amyloid plaque formation. Mitochondrial dysfunction, inflammation, oxidative stress, microvascular abnormalities, and shared degradation pathways are likely only some of the many overlapping pathways between AD and type 2 diabetes, and offer the opportunity to leverage knowledge from diabetes therapeutic development for AD.

1. Therapeutic Approaches to Bioenergetic Dysfunction

While managing diet, promoting exercise, reducing obesity, and managing comorbidities such as diabetes and heart disease may reduce risk of AD, therapeutics are needed that can intervene in the shared underlying pathways of these diseases to significantly abrogate disease progression. Here, we highlight therapeutic strategies that seek to rescue the bioenergenetic dysfunction seen in AD.

One therapeutic strategy to address the hypothesis that shared pathological mechanisms between diabetes and Alzheimer's exist is to repurpose existing diabetes drugs and test them for efficacy in Alzheimer's patients. One of the first classes of diabetes drugs to be tested in AD was thiazolidinediones (TZDs). TZDs were hypothesized to reduce inflammation, improve energy utilization, and be neuroprotective by acting through the target, PPARγ. Two TZD family drugs that are used in the treatment of diabetes, rosiglitazone and pioglitazone, have shown efficacy in AD mouse models through learning and memory improvement, reduced amyloid plaques and microglial activation (Heneka et al., 2005; Pedersen et al., 2006). Clinical trials have shown some encouraging, yet conflicting, results. An experimental study with rosiglitazone found that AD or MCI patients treated with the drug had a reduced rate of cognitive decline compared to placebo (Abbatecola et al., 2010; Watson et al., 2005). However, a larger, randomized, placebo-controlled, double-blind study did not shown any difference on primary outcome measure when compared to placebo (reviewed in Miller et al., 2011). One potential reason for this lack of efficacy is the limited brain penetration of rosiglitazone. The side-effect profile of these drugs is also a concern. In studies of rosiglitizone for diabetes, negative outcomes on disease appeared despite expected positive effects on the surrogate (Action to Control Cardiovascular Risk in Diabetes Study Group et al., 2008). These safety concerns limit TZDs utility for Alzheimer's patients.

While the proposed target of these drugs is PPARγ, they do have other actions and some scientists have argued that the effects on PPARγ result in the negative side-effect profile (edema, cardiac problems) seen with therapy, while the positive therapeutic effects are through a different target, such as the mitochondria (Bolten et al., 2007). In order to test this hypothesis, Metabolic Solutions Development Company designed TZD-related compounds that retain the positive effects of their precursors without interacting with PPARγ. The lead compound, mitoglitazone, interacts with a target in the mitochondria and improves mitochondria function (www.msdrx.com). Initial results in animal model studies were promising, and a Phase II clinical trial is now underway for AD (http://clinicaltrials.gov/ct2/show/NCT01374438). This work also opens the door for the development of second-generation compounds, specifically targeting the mitochondria that would increase cellular energy function.

Mitochondrially targeted antioxidants, such a R(+)-pramipexol, have shown encouraging clinical benefit so far in ALS and PD (Wang et al., 2008) and are now being tested in AD patients (http://clinicaltrials.gov/ct2/show/NCT01388478). Another mitochondria-targeted antioxidant called MitoQ has been shown to reduce oxidative stress and improve cognitive performance and AD-associated pathological outcomes in an AD mouse model (McManus et al., 2011). MitoQ rapidly crosses the blood–brain barrier and neuronal membranes and concentrates several hundred-fold in

mitochondria. Mitochondrial uncouplers are another potential therapeutic strategy to reduce oxidative stress and have been tested primarily in trauma and stroke models (Korde et al., 2005; Pandya et al., 2007).

Metformin is another well-known antidiabetic drug that is being tested for AD. Cellular studies have demonstrated that metformin rescues neuronal insulin resistance and reduces phosphorylation of tau in neuronal cellular models (Gupta et al., 2011). By decreasing levels of insulin in the brain, it may prevent competition for IDE and allow more efficient Aβ degradation. Alternatively, metformin may improve energy utilization and reduce inflammation, which could exert Aβ-independent effects on neuronal function. However, other data from animal model studies imply that metformin may actually increase Aβ and may hasten AD progression (Chen et al., 2009). Jose Luchsinger and team at Columbia University are currently running a clinical trial to assess if metformin treatment will lower insulin levels and improve memory outcomes in MCI patients who are overweight (http://clinicaltrials.gov/ct2/show/NCT00620191).

Glucagon-like peptide-1 (GLP-1) protease-resistant analogs, exendin-4 and liraglutide, have also gained recent interest for their potential neuroprotective properties, in addition to their peripheral efficacy in improving efficiency of glucose uptake (for review, see Holscher (2010)). GLP1 receptors are widely expressed in the brain and can enhance synaptic plasticity and protect neurons from oxidative insults. AD animal model studies have demonstrated reduction in amyloid plaques, and increased neurite outgrowth and dendritic branching with exenden-4 treatment (Li et al., 2010). Clinical trials of liraglutide and exendin-4 for AD are in the works (Hurley, 2012). Other diabetes drugs on the market work by preventing GLP-1 degradation by inhibiting the enzyme dipeptidyl peptidase (IV; DPP-4) and could produce similar beneficial effects as described earlier (Kim & Egan, 2008). DPP-4 inhibitors (e.g., Sitagliptin) have an advantage over exendin-4 and liraglutide. They are small molecules as opposed to biologics and are thus easier to administer and less expensive for patients.

Insulin can also be delivered directly to the brain via an intranasal delivery method. The goal with this treatment would be to increase CNS insulin action and thereby improve learning and memory. In a clinical study of MCI and Alzheimer's patients, intranasal insulin improved cognitive outcomes and prevented decline in FDG-PET signal (Craft et al., 2012). This effect is mediated presumably through activation of insulin receptors in the brain, which are found in particularly high levels in the hippocampus (Havrankova et al., 1978).

Finally, enhancing neuronal energetic status with dietary cofactors can help to prevent cellular energy depletion when neurons are under stress. Creatine treatment, for example, has demonstrated neuroprotective effects in various models of neurodegeneration. Nicotinamide is another possible

treatment and increases the level of NAD+ needed by the mitochondria as an electron/donor acceptor in the electron transport chain to generate ATP (Liu et al., 2008). Administration of 2-deoxyglucose, a glycolysis inhibitor, has also shown potential for improving disease phenotypes relevant to AD. Treatment of the 3x-Tg AD mouse model induced ketogeneis, improved mitochondria function, and decreased AD pathology (Yao et al., 2011).

In summary, these systemic approaches described earlier have significant promise for targeting or preventing multiple aspects of the disease cascade and will likely not only be relevant for AD, but will have the potential to impact multiple diseases of aging.

III. Conclusion

The field is currently awaiting the results of several key Phase III clinical trials that target Aβ. Within the next year, results will be available that will determine the effectiveness of anti-Aβ therapies in mild to moderate AD patients. However, it is unclear if these drugs in current Phase III trials will represent a highly effective safe therapy to slow progression. It is becoming apparent from the biomarker studies using amyloid imaging and CSF measurements that Aβ levels are reaching a plateau 5–10 years before clinical manifestation of the disease (Buchhave et al., 2012). It is therefore questionable that removal of Aβ at this late stage of the disease will show cognitive benefit as the disease cascade leading to cell death has already been initiated. Advances in the field highlight numerous additional pathological manifestations of the disease such as NFT formation, altered protein trafficking and degradation pathways, ApoE regulated pathways, oxidative stress, inflammation, and mitochondrial dysfunction that play a significant role in the disease process. Probing these mechanisms has uncovered a plethora of novel "druggable" targets that have the potential to become viable therapeutic targets for treatment prior to and throughout clinical stages of the disease.

In the opening of this edition, we sought to highlight the innovative therapeutic strategies that are currently progressing through preclinical and clinical development. Ultimately, developing therapeutic strategies targeting these novel pathogenic pathways will open the door to combination therapies that could then be individually modified, based on individual biomarker profile, cognitive status, and adjusted as one moves through the pathological stages of disease. In the end, the Alzheimer's population is not a homogenous one. AD is likely a multifactorial disease and therefore an individualized, multimodal strategy may have the best chance to significantly intervene and halt this devastating disease In the opening of this edition, we sought to highlight the innovative therapeutic strategies that are currently progressing through preclinical and clinical development. Ultimately, developing therapeutic strategies targeting these novel pathogenic pathways will open the door to combination

therapies that could then be individually modified, based on individual biomarker profile, cognitive status, and adjusted as one moves through the pathological stages of disease. In the end, the Alzheimer's population is not a homogenous one. AD is likely a multifactorial disease and therefore an individualized, multimodal strategy may have the best chance to significantly intervene and halt this devastating disease.

Conflict of Interest: The authors declare they have no conflicting interests.

Abbreviations

Aβ	Amyloid beta
Ach	Achacetylcholine
AchR	acetylcholine receptor
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AV	autophagic vacuole
BBB	blood brain barrier
CAA	cerebral amyloid angiopathy
CBD	corticobasal degeneration
CNS	central nervous system
CME	clathrin mediated endocytosis
CVD	cardio vascular disease
EC	enorhinal cortex
FDA	Food and Drug Administration
FAD	familial Alzheimer's disease
FDGPET	fluorodeoxyglucose positron emission spectroscopy
FTD	frontotemporal dementia
FTDP-17	frontotemporal dementia with parkinsonism-17
GWAS	genome wide association study
IND	investigational new drug
IDE	insulin degrading enzyme
ISF	interstitial fluid
LSD	lysosomal storage disease
LTP	long-term potential
MCI	mild cognitive impairment
MRI	magnetic resonance imaging
MS	multiple sclerosis
NFT	neurofibrillary tangle
NSAID	nonsteroidal anti-inflammatory drug
oAβ	oligomeric Aβ
PD	Parkinson's disease
PHFs	paired helical filaments
PSP	progressive supranucleur palsy

SGZ	lsubgranular zone
SNPs	single-nucleotide polymorphism
SVZ	subventricular zone
TBI	traumatic brain injury
TGN	trans-Golgi Network
TZD	thiazolidinediones

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Activation of Protein Kinase C Isozymes for the Treatment of Dementias

Abstract

Memories are much more easily impaired than improved. Dementias, a lasting impairment of memory function, occur in a variety of cognitive disorders and become more clinically dominant as the population ages. Protein kinase C is one of the "cognitive kinases," and plays an essential role in both memory acquisition and maintenance. Deficits in protein kinase C (PKC) signal cascades in neurons represent one of the earliest changes in the brains of patients with Alzheimer's disease (AD) and other types of memory impairment, including those related to cerebral ischemia and ischemic stroke. Inhibition or impairment of PKC activity results in compromised learning and memory, whereas an appropriate activation of certain PKC isozymes leads to an enhancement of learning and memory and/or antidementic effects. In preclinical studies, PKC activators have been shown to increase the expression and activity of PKC isozymes, thereby restoring PKC signaling and downstream activity, including stimulation of neurotrophic activity, synaptic/structural remodeling, and synaptogenesis in the hippocampus and related cortical areas. PKC activators also reduce the accumulation of neurotoxic amyloid and tau protein hyperphosphorylation and support antiapoptotic processes in the brain. These observations strongly suggest that PKC pharmacology may represent an attractive area for the development of effective cognition-enhancing therapeutics for the treatment of dementias.

I. Introduction

Dementia, a lasting impairment of memory function, represents a major challenge to modern medicine. According to Alzheimer's Disease

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International, the total worldwide cost of care for patients with dementias in 2010 is $604 billion (Alzheimer's Disease International, 2010), which is also set to soar as the population ages in the near future. Dementias—including Alzheimer's disease (AD), vascular dementia, dementia with Lewy bodies, and frontotemporal dementia—are memory disorders that are caused by a variety of neural impairments or injuries that lead to compromised cognitive function. There are currently no curative therapeutics for any type of dementia, highlighting an unmet and urgent need for the development of new, cost-effective agents that can target the processes of neural injury that lead to cognitive dysfunction and memory impairment characteristic of dementia.

Cognition, including the formation and retention of memories, results from activity-generated (i.e., acquiring experience and maintaining knowledge of that experience) neuronal Ca^{2+} and other signals that promote gene transcription and protein synthesis in the brain. Protein kinase C (PKC) belongs to a multigene family of phospholipid-dependent serine–threonine kinases, and is part of an essential signaling network in the brain. PKC isoforms are critically involved in modulating synaptic function/transmission; neurite outgrowth/neuronal plasticity; functions of membrane proteins, including enzymes and channels; neuronal metabolism, inflammation, carcinogenesis, proliferation, and gene expression; neuroprotection and neurodegeneration; and behavior, learning, and memory (Alkon et al., 1998; Hama et al., 2004). PKC signaling cascades are impaired or become dysfunctional in many disease processes, and loss of normal PKC signaling may underlie the pathogenesis of various brain disorders, including dementias. Thus, the PKC signaling system represents an important target for discovering new therapeutics for dementias.

II. PKC Signaling System

A. PKC Isoforms

Twelve PKC isoforms have so far been identified in mammals. Based on their homology and sensitivity to activators, they are commonly divided into three subgroups (Fig. 1): (1) classical PKC (cPKC); (2) novel PKC (nPKC); and (3) atypical PKC (aPKC). The number of isoforms differs from other species. For example, in *Aplysia*, at least three isoforms, Apls I, II, and III, have been identified so far.

The cPKC subgroup members contain four homologous domains (C1, C2, C3, and C4) separated by isozyme-specific variable regions (labeled V; Fig. 1), and are activated by Ca^{2+} stimulating factors, such as diacylglycerol (DAG), phosphatidylserine (PS), or other PKC activators. The C-terminal active site contains the C3 and C4 domains and functions as a serine/threonine kinase. The C3 region includes the binding site for adenosine-5'-triphosphate

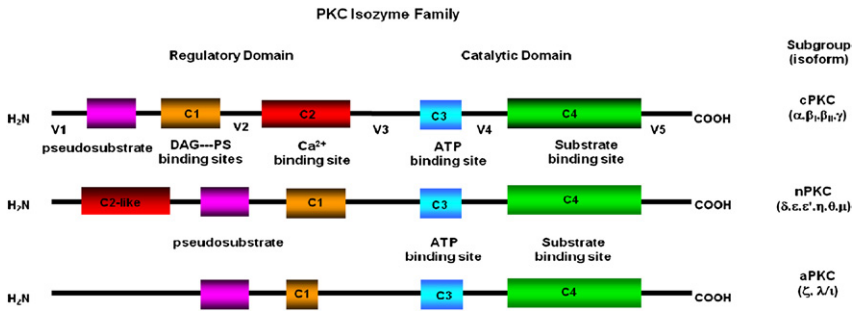

FIGURE 1 **Domain structures of the PKC isoforms.** The homologous domains (C1, C2, C3, and C4) are separated by isoform-unique (variable or V) regions. The C1 domain contains binding sites for diacylglycerol (DAG) and phosphatidylserine (PI). For color version of this figure, the reader is referred to the online version of this book.

(ATP) (as the phosphate donor for phosphotransferase activity), and the C4 region contains the substrate binding site. At the N-terminal, there are two main regulatory domains, the activator-binding C1 domain and the Ca^{2+}-binding C2 domain, which are also involved in membrane association. By contrast, the nPKC subgroup members contain a C2 domain that lacks the acidic Ca^{2+}-binding pocket; as a result, the Ca^{2+}-binding affinity of nPKCs is very low and Ca^{2+} is not required for activation. The aPKC subgroup members lack both the Ca^{2+}-binding site in the C2 domain and one-half of the C1 homologous domain (atypical C1 domain). aPKCs are insensitive to Ca^{2+}, DAG, phorbol esters, and some of the other PKC activators, but they can be activated by PS, arachidonic acid, and ceramide.

One important feature of the PKC isoforms is an N-terminal pseudosubstrate motif near the C1 domain. All of the PKC isoforms but PKCμ (human) and its murine homologue, PKD, contain this motif, which acts as an autoinhibitory domain that binds to the PKC catalytic domain, thereby maintaining an inactive state. Removal of this autoinhibitory fragment is one way by which PKC isoforms can be activated. Upon proteolytic cleavage of the autoregulatory region, the PKC isozymes can be transformed into a persistently active kinase (PKM). For example, PKCδ can be cleaved by caspase-3 to generate a catalytically active kinase (Emoto et al., 1995; Kanthasamy et al., 2003), an event that has been linked to dieldrin-induced dopaminergic degeneration, a potential environmental risk factor for development of Parkinson's disease (Kitazawa et al., 2003).

B. PKC Isoform Activation

PKC activation depends on the presence of required activators, membrane association and translocation, and binding to specific anchoring molecules. The phosphoinositide (PI) signaling pathway is one of the

major cascades that leads to activation of PKC. Stimulation of certain G-protein-coupled receptors activates phospholipase C (PLC, Fig. 2), which hydrolyzes phosphatidylinositol-4, 5-bis-phosphate to form inositol triphosphate (IP_3) and DAG. IP_3 binds to intracellular receptors, causing Ca^{2+} release from the endoplasmic reticulum, whereas DAG binds to and activates most PKC isozymes. The combination of the Ca^{2+} wave and DAG simulate the cPKC isoforms, while DAG alone activates the nPKC and aPKC isoforms. Thus, the concomitant release of intracellular Ca^{2+} release permits activation of all PKC isoforms.

PKC activation also requires membrane association and subcellular translocation. Activated PKCβI, for example, is found inside the nucleus of cardiac myocytes, whereas activated PKCβII is located at the perinucleus and cell periphery. The localization of different PKC isoforms to different areas of the cell appears to involve binding of the activated isoforms to their specific anchoring molecules, the receptors for activated C-kinase (RACKs). RACKs function by selectively anchoring activated PKC isozymes to their respective subcellular sites. They bind only activated PKC

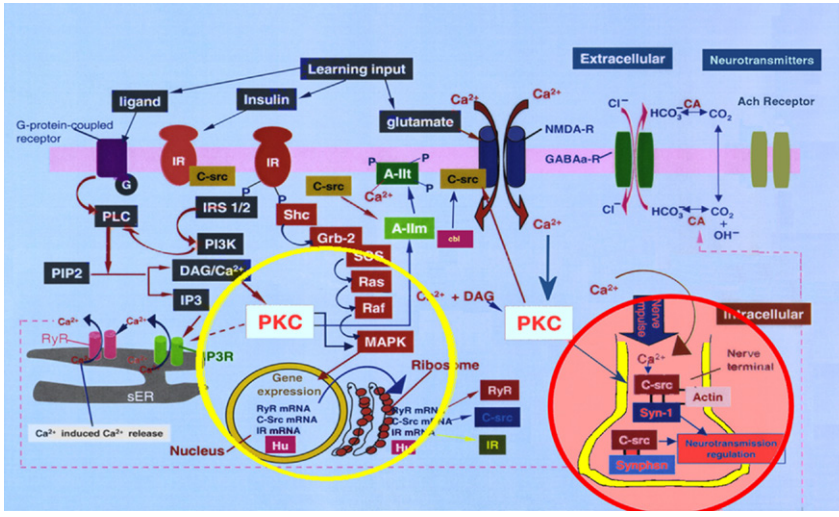

FIGURE 2 **Schematic summary of multimodal drug pathways in memory-enhancing and antidementic therapeutics.** PKC activators may affect neuronal functions through multiple signaling pathways, including regulation of synaptic transmission involved in cognitive processing, membrane channel functions, Ca^{2+} release, gene expression, and protein synthesis. Ach, acetylcholine; cbl, casitas b-lineage lymphoma protein(s); CA, carbonic anhydrase; DAG, diacylglycerol; IP3, inositol triphosphate; IR, insulin receptor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol-4, 5-bis-phosphate; PLC, phospholipase C; RyR, ryanodine receptor; sER, smooth endoplasmic reticulum; SHC, Src homology domain-containing protein(s); Synphsn, synaptophysin. For color version of this figure, the reader is referred to the online version of this book.

but are not necessarily substrates of the enzyme, and PKC binding to RACKs is not mediated via the catalytic domain of the kinase. RACK binding is, however, required for PKC to mediate its cellular responses. Inhibition of PKC binding to RACKs *in vivo* has been shown to inhibit PKC translocation and PKC-mediated functions (Johnson et al., 1996; Ron & Mochly-Rosen, 1995; Smith & Mochly-Rosen, 1992). Aβ oligomers decrease RACK1 distribution in the membrane fraction of cortical neurons (Liu et al., 2011). Peptides that mimic either the PKC-binding site on RACKs or the RACK-binding site on PKC isozymes are isozyme-specific translocation inhibitors of PKCs. For example, an eight amino acid peptide derived from PKCε (εV1-2; Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr) contains a part of the RACK-binding site on PKCε and selectively inhibits PKCε-mediated functions. The structural requirement for PKC isozyme-specific binding by RACKs is of particular interest for the development of PKC isozyme-selective nonpeptide inhibitors and activators.

Depending on the cell types and isoforms involved, activation of PKC isozymes results in phosphorylation of the hydroxyl moiety of serines and threonines within a variety of target proteins. Serine/threonine phosphorylation of a given protein can alter its stability, protein–protein interactions, cellular distribution, or catalytic activity, which in turn propagates signals from the plasma membrane to molecular targets in the cytoplasm and nucleus. One PKC target protein is GAP-43, a growth-associated protein with an approximate molecular weight of 43 kDa.

C. Synaptic and Neuronal Functions of PKC Isozymes

PKC is a known regulator of synaptic functions, including the synthesis, vesicle-refilling, and release of neurotransmitters in cholinergic, γ-aminobutyric acid (GABA)-ergic, dopaminergic, and glutamatergic systems (Dobransky et al., 2004; Malenka et al., 1986; Nicholls, 1998; Okada et al., 2004; Stevens & Sullivan, 1998). PKC also regulates gene expression in mature neurons (Roberson et al., 1999), and the activity and cell surface expression of several plasma membrane proteins, including G-protein-coupled receptors, neurotransmitter transporters (serotonin, dopamine, norepinephrine, glutamate, and GABA), and the Na^+/H^+ antiporter. Activation of PKC enhances Ca^{2+} action potentials, increases neurotransmitter release, and decreases voltage-gated Na^+ currents (Carr et al., 2002; Carr et al., 2003; Chen et al., 2005; Chen, Yu et al., 2006; González et al., 2002) through enhancement of intrinsic slow inactivation gating (Chen et al., 2006) and voltage-dependent K^+ currents (Alkon et al., 1986; Farley & Auerbach, 1986) and Ca^{2+}-activated K^+ currents in the hippocampus, and through inhibition of the delayed rectifier K^+ channel (PKCε, Song et al., 2011). Each of these PKC-mediated synaptic changes are relevant in cognition (Alkon et al., 1986, 1998; Bank et al., 1988; Farley & Auerbach, 1986; LoTurco et al., 1988; Zhang

et al., 2005). PKC activation potentiates synaptic responses in a variety of preparations (Alkon & Rasmussen, 1988; Bank et al., 1988; Kaczmarek, 1987; LoTurco et al., 1988; Stevens & Sullivan, 1998; Zhang et al., 2005).

1. Glutamatergic System

The glutamatergic system, with glutamate as the major excitatory transmitter in the mammalian brain, interacts with PKC signaling pathways. In cultured cerebellar granule neurons, *N*-methyl-D-aspartic acid receptor (NMDAR) activity has been shown to regulate PKC activity (Wang et al., 2004). PKC mediates (–)-epigallocatechin gallate, the main polyphenolic constituent of green tea, to induce Ca^{2+}-dependent glutamate release in the rat cerebral cortex (Chou et al., 2007). PKC also mediates brain-derived neurotrophic factor (BDNF)-mediated modulation of NMDAR subunit 1 in the dorsal horn of the rat spinal cord (Salck et al., 2004). The α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) receptor subunits glutamate receptor (GluR)1 and GluR2 contain type I and type II postsynaptic density protein of 95 kDa/Discs-large/ZO-1 (PDZ) binding motifs, respectively, as does the metabotropic GluR (mGluR)7α. The C-terminus of PKCα has a type I PDZ binding motif, where GluR2 has a type II PDZ binding motif. Both motifs are recognized by the PDZ domain of protein interacting with C kinase 1 (PICK1). The PDZ domain of PICK1 appears to have distinct PKCα and GluR2 binding subsites and PICK1-PKCα-controlled phosphorylation regulates the synaptic expression and function of GluR2 (Dev et al., 2004). Knock-in mice lacking the PDZ-ligand motif of mGluR7α show an impaired PKC-dependent regulation of glutamate release and spatial working memory deficits (Zhang et al., 2008).

It has been shown that PKC activation leads to phosphorylation of GluR 2/3 (at serine 880) in the Purkinje cells. GluR 2/3 phosphorylation appears to be the critical step for parallel fiber long-term depression (LTD; Rekart et al., 2005). Phosphorylation of GluR1 on serine 818 by PKC controls synaptic incorporation of GluR1-containing AMPA receptors into the synapses during long-term potentiation (LTP; Boehm et al., 2006). Postsynaptic inhibition of PKC activity holds AMPARs at the perisynaptic regions, making both LTP and spine expansion labile (Yang et al., 2010). PKC mediates an AMPA receptor subtype switch (from GluR2-lacking [Ca^{2+}-permeable] to GluR2-containing [Ca^{2+}-impermeable] receptors) caused by activation of extrasynaptic NMDARs in mouse cerebellar stellate cells (Sun & Liu, 2007).

In pyramidal neurons of the rat prefrontal cortex, mGluR activity has been shown to enhance NMDAR currents via a PKC-dependent mechanism (Tyszkiewcz et al., 2003). In the perirhinal cortex, mGluR-LTD requires activation of the PKC-PICK1 signaling pathway (Jo et al., 2008). In the hippocampal CA1 pyramidal neurons, mGluR6-containing kainate

receptors are probably involved in PKC-mediated inhibition of the slow after-hyperpolarization (Melyan et al., 2002). Glutamate also desensitizes mGluR5a and mGluR5b via PKC-mediated phosphorylation of mGluR5 at multiple sites (Gereau & Heinemann, 1998).

2. *GABAergic System*

GABA is the major inhibitory neurotransmitter in the adult mammalian brain. In addition to the agents that act on GABA receptors (GABARs) as agonists or antagonists, GABAR currents can be modulated by positive and negative allosteric agents, such as benzodiazepines, barbiturates, neurosteriods, and zinc. PKC phosphorylates several GABAR subunits within their major intracellular domains, changing GABAR functions and their allosteric modulations. Phosphorylation of serine 443 by PKC increases α4 subunit-containing $GABA_AR$ cell surface expression and insertion into the plasma membrane of neurons in the hippocampus, thereby mediating tonic inhibition (Abramian et al., 2010). PKC activation may increase the clearance of GABA from synaptic and extrasynaptic sites into astrocytes (Vaz et al., 2011). PKC activation decreases GABAR function in most cases (Filippova et al., 2000; Krishek et al., 1994; Leidenheimer et al., 1993), but can also increase GABAR currents in some cases (Lin et al., 1994; Lin et al., 1996; Poisbeau et al., 1999). In the hippocampus, PKC activation increases miniature inhibitory postsynaptic current (mIPSC) peak amplitudes in granule cells but have no effect on the mIPSC in CA1 neurons (Poisbeau et al., 1999). In the NT2-N neurons, activation of PKC isozymes results in reduced apparent affinity of diazepam to the GABARs and decreased allosteric enhancement by benzodiazepines (Gao & Greenfield, 2005).

3. *Cholinergic System*

The cholinergic system in the brain plays an important role in learning and memory. Functional deficits in the cholinergic system and neuronal injury are among the earliest detectable abnormalities in neurotransmitter systems in AD. Arachidonic acid stimulates choline acetyltransferase activity through PKC activation (Chalimoniuk et al., 2004). Choline acetyltransferase phosphorylation in neurons is mediated predominantly by PKC at Ser 476 (which is required for phosphorylation at other serine residues to proceed), with PKC activation also increasing phosphorylation at Ser 440 and enhancing choline acetyltransferase activity (Dobransky et al., 2004).

Functional interaction between the cholinergic system and PKC has also been noted. Muscarinic activation of G-protein-coupled receptors leads to stimulation of PLC, which cleaves the membrane phospholipids phosphatidyl-inositol-4,5-bisphosphate to form the PKC activators IP_3 and DAG. As described previously, IP_3 initiates Ca^{2+} release from intracellular stores, and

high Ca^{2+} levels are required for cPKC activation. PKC activation, on the other hand, enhances acetylcholine release from rat hippocampal slices (Chaki et al., 1994). Activation of the presynaptic α7 acetylcholine receptors on the glutamatergic terminals in the CA1 region of the rat hippocampus facilitates glutamate release via an action on PKC (Yamamoto et al., 2005). Based on these observations, another promising approach to developing antidementic therapies would involve targeting PKC-induced presynaptic facilitation (Nishizaki et al., 2000).

4. Dopaminergic System

Dopaminergic activity in the brain is associated with many types of cognition, particularly emotion-associated memory and reward decision-making. The dopamine-mediated enhancement of spike firing in nucleus accumbens shell medium spiny neurons can be prevented by the PKC inhibitor bisindolymaleimide but not by the phospholipase C inhibitor 1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl]-1H-pyrrole-2,5-dione, suggesting a role for the DAG-independent aPKCs (Hopf et al., 2005). In PKCε knockout mice, nicotinic regulation of dopamine release is reduced in the brain reward network (Lee & Messing, 2011), most likely due to a down-regulation of α_6 nAChR subunit mRNA in the ventral midbrain and striatum (Exley et al., 2008). Morphine-induced reward memory, however, may involve the PKCγ isoform (Ping et al., 2012). The protein levels of PKCγ, but not PKCα, βI, βII, and/or ε, were significantly up-regulated in membrane functions of the limbic forebrain obtained from morphine-conditioned mice (Narita et al., 2001). In both porcine aortic endothelial and HeLa cells, PKC activation results in rapid degradation of dopamine transporter ($t_{1/2}$ of approximately 1–2 h; Miranda et al., 2005), through accelerated internalization and probably lysosomal degradation. In C6 glioma cells, internalization is mediated by PKCε, whereas degradation is mediated by PKCα through PI3K (Davis et al., 1998; Gonzalez et al., 2002).

D. Synaptogenesis

Synapses, located on dendritic spines, are polarized structures in which proteins and mRNA become asymmetrically localized. PKC isoforms, including PKCε, are involved in regulation of dendritic spine and synapse structure and function. Activation of PKCε results in synaptogenesis as well as prevents synaptic loss related to brain injury in adult rodents (Hongpaisan & Alkon, 2007; Sun et al., 2008; Sun et al., 2009). PKCε activation leads to an increased expression of BDNF, which initiates complex signaling pathways that modify/repair synaptic structure and function (Adasme et al., 2011). BDNF-induced spine formation and growth require functional RyR (Adasme et al., 2011). At *Drosophila* glutamatergic presynaptic structures,

aPKC regulates the stability of microtubules by promoting their association with the MAP1B-related protein Futsch. At the postsynaptic structure, aPKC regulates the synaptic cytoskeleton by controlling the extent of actin-rich and microtubule-rich areas (Ruiz-Canada et al., 2004). Neurons overexpressing PKMζ, an independent C-terminal domain of PKCζ, exhibit shorter spines, primarily the stubby type, with no differences in terms of spine density, dendritic arborization, or overall viability (Ron et al., 2012). Activation of PKC with 12-myristate 13-acetate, an analogue of DAG, induces rapid morphological plasticity and formation of dendritic lamellae in dendrites of cultured hippocampal neurons (Pilpel & Segal, 2004). PKC inhibitors block neurite outgrowth in retinal axons (Heacock & Agranoff, 1997), dorsal root ganglion neurons (Theodore et al., 1995), sympathetic neurons (Campenot et al., 1994), PC12 cells (Kolkova et al., 2000), and hippocampal organotypic cultures (Toni et al., 1997). These inhibitors also promote dendritic growth in Purkinje cells in cerebellar slice cultures (Metzger & Kapfhammer, 2000) and the extension of dorsal root ganglion cell filopodia (Bonsall & Rehder, 1999).

There is evidence that astrocytes are active participants in synaptic formation and modification (Haydon, 2001). Local astrocytic contact with cultured rat hippocampal neurons via integrin receptors promotes global synaptogenesis (Hama et al., 2004). The astrocyte-neuron contact activates PKC through an arachidonic acid cascade in neurons, triggering excitatory synaptogenesis, a process that can be blocked by inhibitors of both integrins and PKC (Hama et al., 2004).

E. Neuronal Survival

PKC isoforms influence the process of neurite outgrowth or the induction of apoptosis. In general, PKCα, β, ε, and ζ function as suppressors of apoptosis (Khadra et al., 2011), whereas PKC δ and θ are pro-apoptotic (Basu & Pal, 2010). In neuroblastoma cells, for example, PKCε induces neurite outgrowth, whereas PKCδ and PKCθ evoke apoptosis. Plasmalemmal repair/sealing is necessary for survival of damaged neurons, and involves nPKC isoforms (Spaeth et al., 2010). An inhibitor of an nPKC (an nPKCη, pseudosubstrate fragment) decreases the frequency and rate of plasmalemmal sealing in B104 hippocampal cells (Spaeth et al., 2010). There is also evidence that PKC may play an important role in the survival of the spiral ganglion neurons. After deafferentation, activation of PKCβ1 with either phorbol esters or bryostatin-1 induces survival and neurite regrowth and rescues spinal ganglion neurons from cell death (Lallemend et al., 2005).

Nerve growth factor activates phospholipase C-γ, which, upon binding to phosphorylated Tyr^{785} in Trka, is itself phosphorylated and activated, hydrolyzing phosphatidylinositol 4,5-biphosphate to produce DAG and IP_3

and thus activating PKC (Parekh et al., 2000; Toker, 2000). Nerve growth factor activates phosphatidylinositol 3-kinase and PKC in sympathetic neurons, and PKC activation can rescue neurons from apoptosis induced by the withdrawal of nerve growth factor (Favit et al., 1998; Pierchla et al., 2004). PKC may also mediate the neuroprotective effects of estrogen and protect neurons against amyloid beta (Aβ) neurotoxicity (Cordey, Gundimeda, Gopalakrishna, & Pike, 2003). There is a direct neuroprotective effect of PKC against Aβ, demonstrated in culture when the effect of exogenous $A\beta_{42}$ (25 μM, 24 h) is blocked by PKC inhibitors (Cordey et al., 2003). Estrogen activates cPKC and/or nPKC in a variety of cell types nongenomically and can induce translocation of PKCγ through G-protein-coupled estrogen receptors (Qiu et al., 2003).

Kainic acid administration induces upregulation of PKCδ mRNA and protein in the cortex and hippocampus in rats (Kaasinne et al., 2002). Kainate at 50 μM also induces PKCδ translocation from the soluble to the particulate fraction (Jung et al., 2005). Inhibition of PKCδ with rottlerin significantly increases kainite-induced neuronal death, while phorbol 12-myristate 13-acetate attenuates kainite-induced neuronal death (Nitti et al., 2005), suggesting a protective role of PKCδ against kainite toxicity. On the other hand, PKCδ has been found to mediate glycoxidation-dependent apoptosis in NT2 human neurons, since rottlerin protects neurons from glycoxidation-dependent apoptosis (Nitti et al., 2005). Nuclear translocation of PKCζ, a predominantly cytosolic enzyme, is sensitive to caspase-3 inhibition and is believed to mediate NMDA-induced death of cortical neurons. The nuclear translocation of PKCζ induced by NMDA involves caspase-3-dependent PKCζ degradation. Like other aPKC isozymes, PKCζ is not activated by Ca^{2+}, DAG, phorbol esters, or bryostatin; however, it is activated by several lipid mediators, including phosphatidic acid, phosphatidylinositol 3,4,5-triphosphate, arachidonic acid, and ceramide. Aspirin directly inhibits PKCζ activity, thereby protecting against NMDA-induced death of cortical neurons (Crisanti et al., 2005).

III. Memory and Alzheimer's Dementia

PKC isoforms play a critical role in learning and memory. PKCε activation results in an enhanced BDNF activity, which increases hippocampal expression of the Ca^{2+} release channel isoforms ryanodine receptor RyR2, RyR3 (Fig. 2), and PKMζ in the hippocampus (Adasme et al., 2011). PKMζ is believed to play key roles in hippocampal memory maintenance (Shema et al., 2011), through several mechanisms, including persistent inhibition of GluR2-AMPAR removal from the surface of postsynaptic sites (Migues et al., 2010; Yao et al., 2008) and/or alterations

in spine structure (Ron et al., 2012). Overexpressing PKMζ in the rat neocortex enhances long-term memory, whereas a dominant negative PKMζ disrupts memory, even long after memory has been established (Shema et al., 2011).

PKC inhibition or dysfunction, which occurs in neurodegenerative disorders including AD, lead to cognitive impairments in the majority of patients. AD is characterized by a devastating and progressive decline of memory and other cognitive functions. The main histopathological hallmarks of the AD brain are extracellular senile plaques formed by deposits of Aβ peptide and intracellular neurofibrillary tangles consisting of paired helical filaments formed by hyperphosphorylated tau. Aβ occurs in two predominant forms with different COOH-termini, Aβ40 and Aβ42, through cleavage of amyloid precursor protein (APP) by β-secretases and γ-secretases (Fig. 3). Aβ is hydrophobic and prone to aggregation, forming oligomers and plaques. β-secretase cleaves APP at its NH_2-terminus, releasing a soluble NH_2-terminal fragment of approximately 100 kD (sAPPβ) and a 12-kD membrane-bound C99 fragment. On the other hand, cleavage of APP by α-secretase (Postina, 2011), which includes a disintegrin and metalloprotease 10 (ADAM10) as the constitutive α-secretase in neurons (Lichtenthaler, 2011), produces a large soluble fragment and a 10-kD membrane-bound C83 fragment. C99 and C83 can be further cleaved by one or more γ-secretases, resulting in Aβ and a nonpathological p3 peptide, respectively. Synaptotoxic Aβ oligomers inhibit PKC isoforms, decrease RyR2 protein expression, and block BDNF-induced RyR-dependent spine remodeling in hippocampal neurons (Paula-Lima et al., 2011). Tau is a microtubule-associated protein typically found in the axon of neurons and involved in microtubule assembly and the stabilization of growth axons (Mailliot et al., 2000). The hyperphosphorylation of tau prevents its binding to taxol-stabilized microtubules and disrupts microtubule assembly from tau and tubulin (Mandelkow & Mandelkow, 1998). A number of protein kinases and protein phosphatases have been implicated in tau hyperphosphorylation, including glycosynthetase kinase 3β (GSK-3β), phosphokinase A (PKA), phosphokinase C, and Src protein kinase.

The Aβ hypothesis of AD pathogenesis facilitated a strong hope that the ability to halt or reverse AD was possible. However, results from large clinical trials have been disappointing thus far, since patients with dramatic clearance of amyloid showed no clear change in clinical course (Holmes et al., 2008; Schenk et al., 2005; Serrano-Pozo et al., 2010). Patients who received a γ-secretase inhibitor in a recent clinical trial also showed apparently worse cognitive functions (Lilly, 2011). For several reasons, PKC isoforms may represent a potential therapeutic target for the treatment of dementias such as AD. First, PKC isoforms are important signaling molecules in learning and memory (Alkon et al., 1998; Alkon et al., 2007;

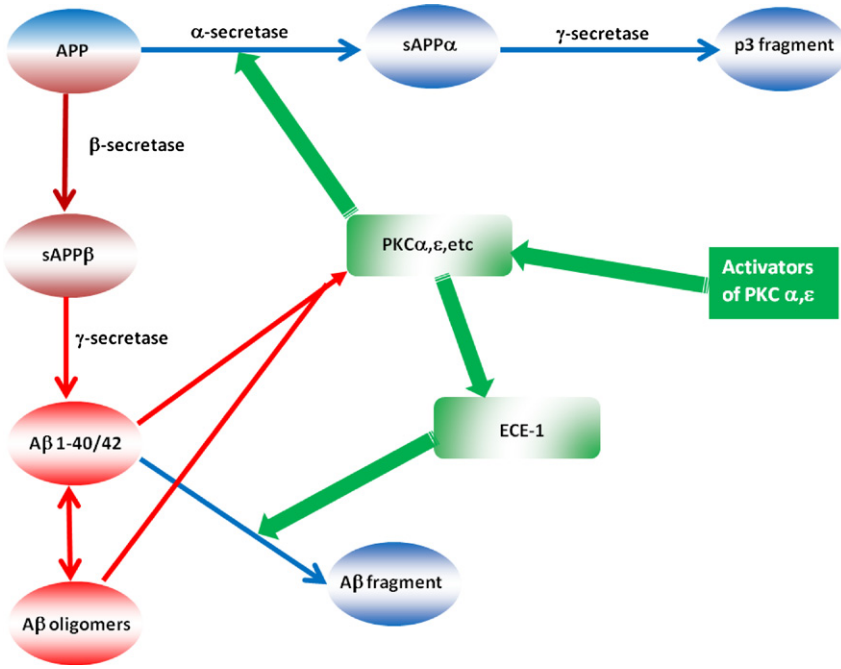

FIGURE 3 **Schematic summary of interaction between Aβ production/clearance and PKC isoform-specific activators.** PKC isoform-specific activators produce antidementic effects through antagonism of the neurotoxic effects of amyloids on PKC, activating α-secretase and endothelin-converting enzyme (ECE). For color version of this figure, the reader is referred to the online version of this book.

Bank et al., 1988; Lorenzetti et al., 2008; Nelson et al., 2008; Sacktor, 2008, 2011; Serrano et al., 2008). PKC is activated by synaptic inputs and intracellular signals that are involved in information processing in cognition, including glutamatergic inputs (Hasham et al., 1997), cholinergic inputs (Chen et al., 2005), serotonergic inputs (Carr et al., 2002, 2003), dopaminergic inputs (Maurice et al., 2001), intracellular calcium and DAG elevation, and other hormonal stimulation (Sato et al., 2004). Memory task learning is associated with PKC immunoreactivity in the principal hippocampal neurons (Van der Zee et al., 1995) and stimulation of muscarinic cholinergic receptors is associated with an increase in PKCγ immunoreactivity (Van der Zee et al., 1992). PKC activation leads to synaptogenesis in the hippocampus (Hongpaisan & Alkon, 2007). Changes in the activity of PKC downstream signaling molecules are also involved. The expression of GAP-43 (Holahan & Routtenberg, 2008), for instance, is up-regulated during spatial learning and memory (Pascale et al., 2004). Transgenic mice overexpressing GAP-43 (Rekart et al., 2004) exhibit enhanced memory in a maze task (Routtenberg et al., 2000), while heterozygous GAP-43

knockout mice have impaired hippocampus-dependent memory (Chung et al., 2003) and contextual fear conditioning (Rekart et al., 2005). PKC signaling cascades are essential for spatial memory acquisition (Colombo et al., 1997; Olds et al., 1989; Olds et al., 1990; Paylor et al., 1991; Paylor et al., 1992; Sun & Alkon, 2005, 2008; Vázquez and de Ortiz, 2004) and consolidation of spatial memory (Bonini et al., 2007), learning and memory of eye blink conditioning (Alkon et al., 1998; Bank et al., 1988; Schreurs et al., 1996; Schreurs et al., 1997; Van der Zee et al., 1997; Wang et al., 2008), olfactory discrimination learning (Olds et al., 1994), conditioned taste aversion (Nunez-Jaramillo et al., 2007; Yasoshima & Yamamoto, 1997), fear memory (Ahi et al., 2004; Levenson et al., 2004; Sacco & Sacchetti, 2010), conditioned avoidance (Jerusalinsky et al., 1994), and drug-associated reward memory (Li et al., 2011; Nimitvilai et al., 2012; Ping et al., 2012). PKC activation with bryostatin-1 induces the *de novo* synthesis of proteins necessary and sufficient for subsequent long-term memory consolidation and enhances memory in *Hermissenda* (Alkon et al., 2005; Kuzirian et al., 2006). Overactivation of PKC may, however, lead to memory impairments, such as working memory in young and old animals (Brennan et al., 2007).

Second, PKC deficiency may underlie many forms of dementias. Expression of PKC isozymes and their functions, especially those in the hippocampus and related brain structures, are plastic and vulnerable to various factors, including stress and neurotoxic amyloid. Inhibition of PKC or impairment of PKC functions consistently leads to deficits in learning and memory (one exception is the report that curcumin-induced PKCδ degradation is associated with enhanced spatial learning in adult and aged rats; Conboy et al., 2009). Intracerebroventricular injection of PKC inhibitors causes marked memory impairment in the passive avoidance task and the water maze task (Takashima et al., 1991). In mice with a deficit in PKCβ, learning of both cued and contextual fear conditioning are impaired, although brain anatomy and hippocampal synaptic transmission, paired-pulse facilitation, and synaptic LTP are all normal (Weeber et al., 2000).

The PKC signaling pathway is impaired in AD (Cole et al., 1988; Govoni et al., 1993; Wang et al., 1994), consistent with evidence that Aβ reduces PKC isozyme levels (Desdouits et al., 1996; Pakaski et al., 2002; Wang et al., 1994). Aβ contains a putative PKC pseudosubstrate domain and can directly inhibit PKC isoforms, including PKCα and PKCε (Lee et al., 2004). Aβ treatment of 1 μM for 1 h induces PKC inhibition that lasts for several hours (Lee et al., 2004). Through binding to PKC, Aβ blocks PKC activation and induces PKC degradation (Cordey et al., 2003), reduces PKC-mediated phosphorylation (Chauhan et al., 1991; Govoni et al., 1993), and decreases PKC membrane translocation (Pakaski et al., 2002). This mechanism of action suggests that the type of interaction between PKC and Aβ

would affect all the PKC isozymes that contain the pseudosubstrate binding site and that the soluble form of Aβ, including oligomers, would be most active. Aβ40, for instance, has been shown to induce translocation of PKC from membrane fraction to cytosol in cultured endothelial cells (Pakaski et al., 2002).

Third, PKCα and ε isoforms regulate the α-processing of APP (Etcheberrigaray et al., 2004; Ibarreta et al., 1999; Jolly-Tornetta & Wolf, 2000; Khan et al., 2009; Kinouchi et al., 1995; Kozikowski et al., 2003; Nelson et al., 2009; Rossner et al., 2001; Yeon et al., 2001; Zhu et al., 2001) and Aβ degradation (Choi et al., 2006; Nelson & Alkon, 2009). α-Processing of APP, mediated by the action of α-secretase, generates a large extracellular soluble APP fragment (sAPPα) and a smaller membrane-bound intracellular fragment, C83. These fragments appear to exhibit no toxic properties to neurons. Evidence has been provided that the administration of bryostatin-1, a partial agonist of cPKC and nPKC isozymes, reduces Aβ40 in the brains of AD transgenic mice and both brain Aβ40 and Aβ42 in AD double-transgenic mice (Kozikowski et al., 2003). Bryostatin-1 at subnanomolar concentrations enhances the secretion of the α-secretase product sAPPα in fibroblasts from AD patients. In APP[V7171] transgenic mice, PKC activation reduces Aβ40 accumulation in the brain (Kozikowski et al., 2003), and in APP transgenic mice, overexpression of PKCε has been shown to selectively increase the activity of endothelin-converting enzyme (ECE, Choi et al., 2006), which degrades Aβ and is expressed in several populations of neurons including the hippocampal cells (Eckman et al., 2001; Funalot et al., 2004). Furthermore, PKC activation inhibits glycogen synthase 3 kinase (Fang et al., 2002; Lavoie et al., 1999), thereby reducing tau protein hyperphosphorylation (Cho & Johnson, 2004).

The combination of memory-enhancing action with reduction in brain amyloid burden and tau protein hyperphosphorylation opens up the possibility for a multitarget strategy with PKC isoform-selective activation that may be an effective therapeutic approach against AD. The therapeutic approach is not expected to interfere with vascular function, as those dysfunctions associated with an enhanced clearance of Aβ from the brains of late-stage AD patients, since the mechanisms of PKC activators, thorough α-secretase and ECE, do not involve an enhanced vascular Aβ clearance. The downside of potent PKC activation may arise due to overactivation of α-processing of APP and/or Aβ degradation, since APP (through an interaction with β1 integrin) and Aβ (including monomers and oligomers) may function in normal physiology by mediating neuronal adhesion and migration (Siemes et al., 2006; Young-Pearse et al., 2007), and promoting neurite outgrowth (Hoareau et al., 2008; Hoe et al., 2009; Perez et al., 1997; Small et al., 1999) and synaptic plasticity and memory (Puzzo et al., 2008).

IV. Ischemic Dementia

It has been well established that ischemia and hypoxia dramatically impair cognitive function. Not only are synapses and neural structures directly impaired by ischemia, but also the process of acquiring and maintaining knowledge that requires energy. PKC is involved in synaptic dysfunction and memory impairments in patients surviving ischemic events (cerebral ischemia, cardiac arrest, etc.; Perez-Pinzon et al., 2005). Global cerebral ischemia triggers DAG kinase (DGK)ξ translocation from the nucleus to the perikaryal cytoplasm of CA1 pyramidal cells during the very early phase of an ischemic insult, probably resulting in a sustained increase in DAG levels and PKC activity in the nucleus (Ali et al., 2004). Cerebral ischemia increases PKCδ mRNA and protein levels in the cortex and hippocampus (Koponen et al., 2000; Miettinen et al., 1996). The increased PKCδ expression in the penumbral area may be responsible for delayed neuronal damage (Phan et al., 2002). Activation of PKCδ by cerebral ischemia results in cytochrome C release from the mitochondria and apoptosis (Dave et al., 2011). A selective PKCδ peptide inhibitor, for example, has been found to reduce cellular injury in a rat hippocampal slice model of cerebral ischemia. The inhibitor decreased infarct size *in vivo* in rats with transient middle cerebral artery occlusion when administered at the onset, at 1 h, or at 6 h of reperfusion (Bright et al., 2004). Hypoxia activates PKC, leading to phosphorylation of NMDA NR1 subunits and an enhancement of GluR activity and Ca^{2+} influx (Bickler et al., 2004). In acutely dissociated rat CA1 neurons, oxygen and glucose deprivation after removal of extracellular Ca^{2+} can still activate PKC through endogenous Ca^{2+} release (Larsen et al., 2004), suggesting that a brief period of cerebral ischemia without exposure to excitotoxicity is sufficient to activate PKC.

On the other hand, PKCε mediates ischemic tolerance (Liu et al., 2012). Activation of PKCε, as a vital part of adenosine/ NMDA-activated signal transduction pathway, protects neurons from ischemia-reperfusion injury (Di-Capua et al., 2003; Raval et al., 2003) and oxygen-glucose deprivation damage, whereas selective inhibition of PKCβI enhances astrocyte cell death induced by oxygen-glucose deprivation (Wang et al., 2004). PKCε phosphorylates and inhibits GSK3β, the inhibition of which during reperfusion promotes glycogen synthesis, thus decreasing glycolysis and associated harmful H^+ production during reperfusion (Takeishi et al., 2000). Ischemic preconditioning is associated with PKCε-mediated phosphorylation of the mitochondrial K^+_{ATP} channels (Raval et al., 2007) and increased synaptosomal mitochondrial respiration (Dave et al., 2008). PKCε knockout mice lose the preconditioning effect of ischemia-reperfusion (Raval et al., 2007). Electroacupuncture pretreatment has been shown to produce PKCε-mediated anti-apoptosis and rapid tolerance to focal cerebral ischemia (MCAO) in rats (Wang et al., 2011). Postischemic activation in rats with intermittent doses of bryostatin-1, which is a relatively specific activator of PKCε, has been found

to restore neurotrophic activity and synaptogenesis in the hippocampus and spatial learning and memory performance after global cerebral ischemia (Sun et al., 2008, 2009). The protective action of bryostatin-1 may involve alterations in cerebral blood flow (Della-Morte et al., 2011). Thus, an appropriate activation of PKC isozymes with targeted PKC activators may represent an effective therapeutic approach to stroke/ischemia-reperfusion injury and associated memory impairment, through activation of ischemic preconditioning responses and enhancement of neurotrophic activity, synaptogenesis and synaptic remodeling.

Female animals are less vulnerable to ischemia-induced neuronal damage (Alkayed et al., 1998; Zhang et al., 1998) and estrogen treatment protects the brain from experimental stroke (McCullough & Hurn, 2003; Yang et al., 2000). PKCε preferentially stimulates the transcriptional activity of estrogen-related receptor α, which regulates mitochondrial homeostasis (Lu et al., 2011). Transient unilateral middle cerebral artery occlusion (90 min) followed by 22.5 h reperfusion has been shown to produce smaller total infarct size in C57BL/6 female mice than in the male mice, but no difference was observed in PKCγ knockout mice (Hayashi et al., 2005). Injection of estrogen (i.p.) after the start of reperfusion can significantly reduce the infarct volume in males but again, the protective effect was attenuated in PKCγ-knockout mice (Hayashi et al., 2005). These data suggest that the neuroprotective effect of estrogen against cerebral ischemia is present in rodents; however, in humans, the clinical evidence for stroke prevention with hormone replacement therapy remains inconclusive.

V. Conclusion

PKC isoforms are distributed in neuronal structures and involved in a broad range of vital functions (Brenner et al., 2004; Lee et al., 2006; Pascale et al., 2007). PKC is ubiquitously and densely expressed in the brain (Saito et al., 1988) and activated by Ca^{2+}, phospholipids and DAG, phorbol esters, and other PKC activators.

PKC activators, such as DAG, arachidonic acid, phorbol esters, bryostatins, aplysiatoxins, and teleocidins, bind to a hydrophilic cleft in a largely hydrophobic surface of the C1 domains, resulting in an enhanced hydrophobicity of the surface and promoting the interaction between the C1 domain and the phospholipid bilayer of the cell membranes and driving removal of the pseudosubstrate region from the catalytic site of the enzyme. When dosed appropriately, these activators may produce memory-enhancing and antidementic effects.

In addition to its inhibitory action on GSK3β, the PKCα and ε activator bryostatin-1 increases soluble APP fragment production through α-secretase in tissues obtained from AD patients and significantly improves spatial

learning and memory in rats. These actions have obvious therapeutic value for the treatment of AD amyloidosis and associated dementias. The availability of bryostatin is limited by their low natural abundance and difficulties with synthesis. 8-[2-(2-Pentylcyclopropyl-methyl)-cyclopropyl]-octanoic acid (DCP-LA), on the other hand, has been shown to selectively activate PKCε, possibly through binding to the PS binding site (Kanno et al., 2006; Nelson et al., 2009). These agents can activate the PKC isoforms and reverse Aβ-mediated neurotoxic effects in the presence of a high Aβ load (Hongpaisan et al., 2011; Khan et al., 2009), raising the possibility that they may be effective at all stages of the disorder. In addition, these agents can achieve therapeutic effects through oral administrations (at different dosing; Sun et al., unpublished observations). The potential values of PKC activators, especially isoform-specific activators, as antidementic therapeutics rely mainly on the following three pharmacological profiles:

1. Functional restoration/facilitation of the PKC signal cascades that are critically involved in memory acquisition and maintenance
2. Reduction of the pathological factors—Aβ accumulation and tau protein hyperphosphorylation—that are associated with or underlie dementia
3. Activation of endogenous neurorepair/protective mechanisms and synaptogenesis against neurodegenerative disorders and ischemic damage

The desired pharmacological profile for the treatment of dementias includes a selective activation of PKC isozymes (PKCε and probably PKCα, but not PKCδ), without inducing significant degradation. Activation of PKC, however, is also involved in the formation of conditioned cue-provoked cocaine memory (Lai et al., 2008), reward memory related to comorbid nicotine and alcohol addictions (Lee & Messing, 2011), and maintenance of persistent pain and pain hypersensitivity (Laferriere et al., 2011). Memories of negative events and unwanted fear may also be enhanced. It remains to be determined whether the involvement of PKC in methamphetamine-induced, long-lasting astrocytic activation and behavioral sensitization (Narita et al., 2005) would jeopardize clinical use of these agents as therapeutics for certain patients. In addition, inhibition of PKCα and β1 may underlie curcumin-induced attenuation of diabetic nephropathy (Soetikno et al., 2011). PKC activity is significantly increased in synpatosomal samples isolated from the forebrain, midbrain, and hindbrain of spontaneously hypertensive rats (Hughes-Darden et al., 2001), responsible for the enhanced basal neural activity in the anterior hypothalamic area (Kubo & Hagiwara, 2005). Many of the potential adverse and side effects may be reduced through the development of PKC region/isozyme-specific agents, such as DCP-LA and its derivatives, in the future.

Conflict of Interest: The authors have no conflicts of interest to declare.

Abbreviations

Aβ	amyloid β-peptide
AD	Alzheimer's disease
ADAM	a disintegrin and metalloprotease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate
aPKC	atypical PKC
APP	amyloid precursor protein
BDNF	brain-derived neurotrophic factor
cPKC	conventional PKC
DAG	diacylglycerol
ECE	endothelin-converting enzyme
GAP-43	growth-associated protein with an approximate molecular weight of 43 kDa
GluR	glutamate receptor
GSK-3β	glycogen synthetase kinase 3β
IP_3	inositol triphosphate
LDP	long-term depression
LTP	long-term potentiation
mGluR	metabotropic GluR
mIPSC	miniature inhibitory synaptic current
NMDAR	*N*-methyl-D-aspartic acid receptor
nPKC	novel PKC
PDZ	ostsynaptic density protein of 95 kDa/Discs-large/ZO-1
PI	phosphoinositide
PICK	protein interacting with C kinase
PKA	phosphokinase A
PKC	protein kinase C
PLC	phospholipase C
PS	phosphatidylserine
RACK	receptor for activated C-kinase
RyR	ryanodine receptor
V	variable (region)

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Striatal-Enriched Protein Tyrosine Phosphatase in Alzheimer's Disease

Abstract

Alzheimer's disease (AD) is the most common form of dementia among the elderly, affecting millions of people worldwide and representing a substantial economic burden. AD is a progressive disease associated with memory loss and impaired cognitive function. The neuropathology is characterized by cortical accumulation of amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques are small, aggregated peptides called beta amyloid (Aβ) and NFTs are aggregates of hyperphosphorylated Tau protein. Because Aβ disrupts multiple intracellular signaling pathways, resulting in some of the clinical symptoms of AD, understanding the underlying molecular mechanisms has implications for the diagnosis and treatment of AD. Recent studies have demonstrated that Aβ regulates striatal-enriched protein tyrosine phosphatase (STEP) (PTPN5). Aβ accumulation is associated with increases in STEP levels and activity that in turn disrupts glutamate receptor trafficking to and from the neuronal membrane. These findings indicate that modulating STEP levels or inhibiting its activity may have beneficial effects for patients with AD, making it an important target for drug discovery. This article reviews the biology of STEP and its role in AD as well as the potential clinical applications.

I. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disorder in people aged 65 years and older and its prevalence is increasing as the population

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ages. It is characterized by irreversible and progressive loss of cognitive function. Clinical symptoms include mild to severe memory loss, problems with cognition and behavior, and gradual losses in the activities of daily living (Castellani et al., 2010). At cellular level, AD is associated with gradual synapse loss, followed by severe neurodegeneration in the brain areas related to cognitive functions. None of the available pharmacological treatments for AD provide more than temporary relief from the relentless decline in cognitive and daily function. It is critically important to understand the pathophysiology of this disease at the molecular level in order to develop new pharmacological treatments.

In AD, brain regions involved in cognitive functions such as hippocampus, cortex, and amygdala show pronounced pathological alterations. Postmortem studies of AD brains have established the neuropathological hallmark of this disease: the accumulation of amyloid plaques and neurofibrillary tangles (NFTs). Beta amyloid (Aβ) peptides accumulate during the course of the disease and contribute to synaptic dysfunction (Hardy & Selkoe, 2002; Haass & Selkoe, 2007). Transgenic mice that overproduce Aβ (Philipson et al., 2010) show that the Aβ produced at the onset of the illness disrupts synaptic function and contributes to cognitive impairment early in the disease process (Hsiao et al., 1996; Jacobsen et al., 2006). The toxic effect (Terry et al., 1991) of Aβ on synapse function is confirmed by its ability to inhibit long-term potentiation (LTP), induce aberrant changes in the synaptic networks, cause synapse loss, and disrupt cognitive functions in animal models (Lacor et al., 2007; Palop & Mucke, 2010; Shankar et al., 2008; Walsh et al., 2002).

Striatal-enriched protein tyrosine phosphatase (STEP) is a brain-enriched tyrosine phosphatase (Lombroso et al., 1991). Accumulating evidence implicates STEP in the pathophysiology of AD. STEP regulates several synaptic events including glutamate receptor trafficking, which plays a crucial role in learning and memory (Baum et al., 2010; Fitzpatrik & Lombroso, 2011a; Goebel-Goody et al., 2012). Recent findings indicate that Aβ peptides generated during the course of disease regulate the function of STEP by up-regulating its activity and protein levels through different mechanisms. Increased STEP activity and protein levels lead to excessive internalization of glutamate receptors both, NMDARs (*N*-methyl-D-aspartate receptors) and AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) from the neuronal membrane, which is thought to be responsible for the synaptic changes associated with cognitive and memory deficits in AD (Kurup et al., 2010a; Snyder et al., 2005; Zhang et al., 2010). The role of STEP in these events has been confirmed in AD mouse models by several *in vitro* and *in vivo* studies, as well as behavioral and electrophysiological studies. This chapter reviews what we know about STEP beginning with its discovery and ending with recent demonstrations of its role in the pathophysiology of AD.

II. Striatal-Enriched Protein Tyrosine Phosphatase (STEP)

Protein kinases and protein phosphatases regulate a great variety of cellular pathways including cell division and higher order brain functions including learning and memory (Mayford, 2007). A major class of protein kinases is those that phosphorylate their substrates at tyrosine residues to initiate or modulate intracellular events. Protein tyrosine phosphatases (PTPs) oppose these activities by dephosphorylating the tyrosine residues, thus playing a major role in cellular signaling. Although STEP was initially discovered as a protein enriched in the striatum (Lombroso et al., 1991), it is distributed in other regions of brain including the cortex and hippocampus (Lombroso et al., 1993). STEP is not present in the cerebellum; here it is substituted by a homologous PTP called STEP-like PTP (Shiozuka et al., 1995). Another closely related PTP expressed in immune cells termed "HePTP" (Hematopoietic Protein Tyrosine Phosphatase) shares sequence homology with STEP (Adachi et al., 1992).

STEP is an intracellular tyrosine phosphatase encoded by the *ptpn5* gene. It exists as two major isoforms, $STEP_{61}$ and $STEP_{46}$, named after their protein mobility in SDS-PAGE (Boulanger et al., 1995; Bult et al., 1997) (Fig. 1). The distribution of these two STEP isoforms varies within different brain regions. $STEP_{61}$ is present in the cortex, hippocampus, and striatum, whereas all isoforms are present in the striatum (Boulanger et al., 1995; Bult et al., 1996). The expression pattern of STEP isoforms changes during development (Raghunathan, Matthews, Lombroso, & Naegele, 1996). Rodent studies indicate that $STEP_{61}$ is expressed at birth and its expression continues throughout adulthood, whereas $STEP_{46}$ first appears at postnatal day 6 and progressively increases until adulthood, indicating that the expression of STEP is developmentally regulated, although a specific role of STEP during

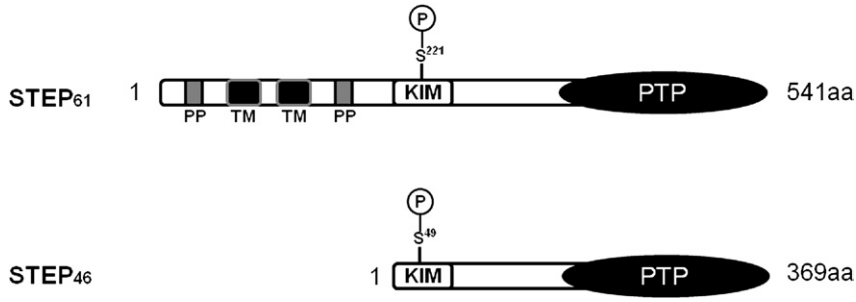

FIGURE 1 Domain structure of STEP isoforms. STEP occurs as two major isoforms: $STEP_{61}$ (541 amino acid residues) and $STEP_{46}$ (369 amino acid residues). Both these isoforms have conserved PTP catalytic domain at C-terminal and KIM (kinase interacting motif) domain. KIM domain has a conserved serine residue, which gets phosphorylated by protein kinase A (PKA), denoted as Ser^{221} in $STEP_{61}$ and Ser^{49} in $STEP_{46}$. $STEP_{61}$ isoform has additional N-terminal region (172 amino acids) containing polyproline-rich domains (PP) and transmembrane domains (TM).

later stages of life or in cognitive deficits that occur with aging has not yet been examined (Okamura et al., 1997; Raghunathan et al., 1996). $STEP_{46}$ is primarily a cytosolic protein, whereas $STEP_{61}$ is targeted to membrane compartments (e.g., endoplasmic reticulum, Golgi bodies, and endosomes) and postsynaptic densities (Goebel-Goody et al., 2009; Oyama et al., 1995).

A. Domain Structure

Both $STEP_{61}$ and $STEP_{46}$ isoforms share a common conserved PTP catalytic domain at their C-terminal region consisting of a conserved sequence ([I/V]HCxAGxxR[S/T]G) that contains a critical cysteine residue required for phosphatase activity. Mutation of the cysteine residue results in a catalytically inactive variant. All STEP isoforms contain a kinase interacting motif (KIM), which is required for the interactions between STEP and its physiological substrates (Bult et al., 1996). The KIM contains a critical serine residue; phosphorylation of this residue by protein kinase A (PKA) prevents STEP from interacting with and dephosphorylating its substrates (Paul et al., 2000; Paul et al., 2003).

$STEP_{61}$ differs from $STEP_{46}$ by an additional 172 amino acid residues in the N-terminal region. This sequence contains two hydrophobic domains that are essential for targeting $STEP_{61}$ to the neuronal membrane, including postsynaptic densities. The N-terminal region of $STEP_{61}$ also contains two polyproline-rich domains and PEST motifs, which are rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (Boulanger et al., 1995; Bult et al., 1996; Oyama et al., 1995). The N-terminal polyproline domain is required for the association of $STEP_{61}$ with Fyn kinase (Nguyen, et al., 2002), while the second polyproline domain is necessary for the interaction of $STEP_{61}$ with Pyk2 (Xu et al., 2012). The PEST sequences in several proteins are known to mediate rapid degradation (Shumway et al., 1999; Spencer et al., 2004), they may also serve as recognition motifs for proteolytic cleavage or ubiquitination of STEP under certain physiological conditions. Additional STEP members like ($STEP_{38}$ and $STEP_{20}$ exists, but their functions are not known. These isoforms do not contain conserved PTP domain and they are catalytically inactive (Bult et al., 1996; Sharma, et al., 1995). The recently resolved crystal structure of STEP shows some distinctive features compared to other PTPs (Eswaran et al., 2006), including a unique open conformation that is critical for PTP catalysis (WPD loop). This structure may prove useful in the search for small, specific STEP inhibitors.

B. STEP Regulation

STEP is regulated by several mechanisms including phosphorylation, ubiquitination, proteolytic cleavage, oligomerization, and local translation

(Deb, et al., 2011; Kurup et al., 2010a; Paul et al., 2000; Xu et al., 2009; Zhang et al., 2008). Recent work shows that PKA phosphorylation and ubiquitination of STEP play a role in AD (Kurup et al., 2010a; Snyder et al., 2005). Both events decrease STEP activity in neurons. PKA phosphorylation of a regulatory serine residue within the KIM domain interferes with the ability of STEP to interact with its substrates (Paul et al., 2000). Ubiquitination rapidly removes $STEP_{61}$ from synaptic sites and promotes degradation by the proteasome (Kurup et al., 2010a; Xu et al., 2009). A model has thus emerged that STEP normally opposes the development of synaptic strengthening by inactivating enzymes that facilitates this process. STEP must be inactivated at synaptic sites, either by phosphorylation within the KIM domain or by rapid degradation for synaptic plasticity and learning to take place (Braithwaite et al., 2006b; Fitzpatrick & Lombroso, 2011; Goebel-Goody et al., 2012). Thus, events that disrupt STEP inactivation would oppose synaptic strengthening.

1. Phosphorylation

Phosphorylation is an important form of posttranslational modification that regulates various intracellular signaling pathways. Both $STEP_{61}$ and $STEP_{46}$ isoforms are phosphorylated by PKA. PKA-mediated STEP phosphorylation was initially discovered after dopamine receptor (D1R) activation (Paul et al., 2000). Dopamine (DA) D1 receptor (D1R) stimulation activates PKA leading to phosphorylation of both $STEP_{61}$ and $STEP_{46}$ at a conserved serine residue (designated ser^{221} in $STEP_{61}$ and ser^{49} in $STEP_{46}$). Phosphorylation at these serine residues results in steric interference, preventing STEP from interaction with its substrates (Paul et al., 2000). PKA also opposes the dephosphorylation of STEP by inhibiting the phosphatase PP1 through a DARPP-32-mediated pathway. PKA phosphorylates DARPP-32 at Thr^{34}, and this phosphorylated form of DARPP-32 acts as a potent inhibitor for PP1 and blocks its activity (Greengard et al., 1999). By initiating these parallel events PKA stabilizes the phosphorylated and inactive forms of STEP (Valjent et al., 2005). PKA phosphorylates another unique serine residue ser^{160} in $STEP_{61}$ at its N-terminal region, although the functional significance is not known.

$STEP_{61}$ phosphorylation at ser^{221} is reduced in AD mouse models and in neuronal cultures treated with Aβ (Kurup et al., 2010a; Snyder et al., 2005). Such conditions would increase the ability of STEP to interact with and dephosphorylate its substrates. Dephosphorylation of these serine residues is mediated by calcineurin (PP2B)/PP1 pathway, favoring its interaction with substrates (Snyder et al., 2005; Valjent et al., 2005). As discussed in the following sections, Aβ peptide binds to α7 nicotinic acetylcholine receptors (α7 nAChRs) that in turn lead to the activation of PP2B and STEP dephosphorylation (Snyder et al., 2005).

2. *Ubiquitination*

The ubiquitination of target proteins involves the covalent attachment of ubiquitin to the substrate and often leads to proteasomal degradation of the protein. The ubiquitin–proteasome system (UPS) plays an important role in cellular protein recycling and has been implicated in several pathological processes including cancer and neurodegenerative disease (Hegde & Upadhya, 2011; Yi & Ehlers, 2007). $STEP_{61}$ is a target for ubiquitin-mediated proteasomal degradation. $STEP_{61}$ levels are increased in cortical neurons treated with Aβ (Kurup et al., 2010a). The increase in $STEP_{61}$ is insensitive to transcription or translation inhibitors, suggesting that $STEP_{61}$ accumulation occurs when normal degradation is blocked. This work led to the isolation of STEP-ubiquitin conjugates from cells treated with proteasome inhibitors, suggesting that $STEP_{61}$ is a direct substrate for ubiquitin conjugation and proteasomal degradation. Together, this work indicates that Aβ-mediated inhibition of the proteasome leads to $STEP_{61}$ accumulation (Kurup et al., 2010a).

STEP is differentially regulated by synaptic and extrasynaptic NMDARs (Xu et al., 2009). These receptors are localized in distinct compartments on the neuronal membrane where they initiate signaling pathways when activated by glutamate (Hardingham & Bading, 2010; Ivanov et al., 2006). Synaptic NMDAR activation is coupled to extracellular-regulated kinase (ERK) activation and is involved in synaptic strengthening and neuronal survival (Hardingham et al., 2002). In contrast, extrasynaptic NMDAR activation is linked to p38 activation and cell death pathways. When synaptic NMDARs are stimulated, $STEP_{61}$ is ubiquitinated and rapidly degraded from synaptic sites by the UPS pathway (Xu et al., 2009). STEP degradation is required for sustained ERK activation. Activated ERK phosphorylates several synaptic and cytoplasmic proteins, and is translocated to the nucleus where it phosphorylates and activates transcription factors such as CREB and Elk-1 that are involved in spine remodeling (Thiels & Klann, 2001).

C. STEP Substrates

1. *Mitogen-Activated Protein Kinase*

The mitogen-activated protein kinase (MAPK) family of proteins consists of several enzymes that activate signaling pathways to regulate cellular differentiation, cell survival and synaptic plasticity (Sweatt, 2004; Thomas & Huganir, 2004). The MAPK family of extracellular signal-regulated kinases (ERK1/2) and p38 are both STEP substrates. STEP dephosphorylates regulatory tyrosine residues in the activation loop of ERK1/2 (Tyr^{204} in ERK1 and Tyr^{187} in ERK2) and p38 (Tyr^{182}), leading to inactivation of these proteins (Munoz et al., 2003; Paul et al., 2003; Xu et al., 2009).

The ERK1/2 signaling pathway regulates synaptic plasticity by post-translational modification of synaptic proteins and by initiating nuclear transcription in neurons (Davis, Vanhoutte, Pages, Caboche, & Laroche, 2000). Activation of ERK1/2 also initiates the local translation of mRNAs targeted to synapses, as well as promoting neurotransmitter release from presynaptic axon terminals (Gelinas et al., 2007; Jovanovic et al., 2000). These events lead to changes in dendritic morphology required for the induction and maintenance of synaptic plasticity (Thomas & Huganir, 2004). Both $STEP_{61}$ and $STEP_{46}$ dephosphorylate the regulatory tyrosine residue of ERK2, thereby playing a role in regulating the duration of ERK signaling (Paul et al., 2003).

The impact of STEP on these mechanisms has been demonstrated with a membrane-permeable TAT (transactivator of transcription)-STEP-cysteine to serine isoform. TAT-STEP (CS) is an inactive variant of STEP, which binds to but does not release its substrates, as release depends on dephosphorylation (Snyder et al., 2005; Tashev et al., 2009). Infusion of TAT-STEP (CS) into the lateral amygdale of rats had no effect on the acquisition of Pavlovian fear conditioning but blocked the consolidation of these memories, suggesting that the inhibition of ERK-mediated downstream events is required for memory consolidation (Paul et al., 2007). Further insights into the role of STEP in regulating ERK2 are provided by studies involving the STEP knock out mouse (STEP KO). The hippocampus of STEP KOs show increased activation of ERK1/2 and its downstream phosphorylation targets, CREB and ELK transcription factors (Venkitaramani et al., 2011; Venkitaramani et al., 2009). Furthermore, STEP KO mice perform better in hippocampus-dependent memory tasks compared to wild-type littermates, which is consistent with prolonged ERK1/2 activation (Venkitaramani et al., 2011).

p38 is a second member of the MAPK family that is dephosphorylated and inactivated by STEP (Munoz et al., 2003; Poddar et al., 2010; Xu et al., 2009). p38 activation plays a role in NMDAR-mediated neuronal excitotoxicity and initiates cell death pathways (Bossy-Wetzel et al., 2004; Semenova et al., 2007). Excess glutamate release results in p38 phosphorylation by preferential activation of extrasynaptic GluN2B-containing NMDARs, and p38 in turn phosphorylates several key proteins involved in cell death pathways (Poddar et al., 2010; Xu et al., 2009). Extrasynaptic NMDAR activation results in $STEP_{61}$ cleavage by calpain, resulting in a nonfunctional isoform, $STEP_{33}$. $STEP_{33}$ lacks an intact KIM domain and does not interact with its substrates, including p38. This leads to the sustained activation of p38 and favors p38-mediated cell death pathways. Preventing $STEP_{61}$ cleavage with a peptide corresponding to the calpain cleavage site protects neurons from glutamate-mediated excitotoxicity (Xu et al., 2009). This neuroprotective effect is accompanied by decreased $STEP_{61}$ cleavage and decreased p38 phosphorylation at its regulatory tyrosine residue.

In summary, ERK2 and p38 are differentially regulated by $STEP_{61}$, depending on whether synaptic or extrasynaptic NMDARs are activated. Stimulation of synaptic NMDARs results in the degradation of $STEP_{61}$ by the UPS, favoring the development of synaptic plasticity and neuronal survival. Extrasynaptic NMDAR stimulation results in $STEP_{61}$ cleavage by calpain, p38 activation, and promotes cell death. Both of these events are implicated at different stages of in AD. The role of STEP in regulating p38-mediated pathway and neuronal death in later stage of AD is under investigation.

2. *Fyn*

Fyn is a member of Src family of tyrosine kinases that was originally identified as a proto-oncogene regulating cellular growth (Semba et al., 1986). It is a nonreceptor tyrosine kinase associated in part with the cytoplasmic side of the plasma membrane. Fyn is targeted to the postsynaptic density and regulates neuronal signaling, including synaptic plasticity (Ali & Salter, 2001; Husi et al., 2000; Walikonis et al., 2000). Fyn activity is regulated by its own tyrosine phosphorylation; it is activated by autophosphorylation at a tyrosine residue (Tyr^{420}), whereas phosphorylation by C-terminal Src kinase (Tyr^{531}) leads to Fyn inactivation (Sun et al., 1998; Superti-Furga, et al., 1993). One role that Fyn plays in synaptic strengthening is to participate in the trafficking of NMDARs (Kohr & Seeburg, 1996; Lau & Huganir, 1995). Fyn phosphorylates GluN2B subunit at Y^{1472} residue in a conserved (YEKL) motif of, resulting in NMDAR insertion into membrane (Nakazawa, Komai, & Tezuka et al., 2001; Roche et al., 2001).

STEP binds to Fyn by interacting with the first polyproline domain and the KIM domain. STEP opposes Fyn activation by dephosphorylating the tyrosine residue (Tyr^{420}) (Nguyen et al., 2002). STEP KO mice have increased Fyn tyrosine phosphorylation at Tyr^{420} and increased phosphorylation of NR2B GluN2B subunit at Tyr^{1472}. Moreover, STEP KO mice show increased surface NMDAR levels, enhanced theta-burst LTP in hippocampal slices, and improved hippocampus-dependent memory (Venkitaramani et al., 2011; Zhang et al., 2010). Together, these findings suggest that STEP regulation of Fyn contributes to suppression of synaptic plasticity and memory consolidation.

3. *Glutamate Receptors*

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system. It is involved in several physiological processes including synaptic plasticity, learning and memory, and several pathological processes that promote excitotoxicity through excessive glutamate release (Lau & Tymianski, 2010; Riedel et al., 2003). Glutamate binding to metabotropic glutamate receptors (mGluR) mediates cellular signaling via G-protein

coupled pathways. Glutamate activation of ionotropic glutamate receptors leads to ion influx and changes in the postsynaptic membrane potential, which in turn activates signaling cascades inside neurons (Mayer & Armstrong, 2004; Traynelis et al., 2010). As mentioned earlier, two major classes of ionotropic receptors regulated by STEP include the NMDARs and AMPARs, both of which play major roles in synaptic plasticity and learning and memory (Pelkey et al., 2002; Snyder et al., 2005; Zhang et al., 2008). In AD, Aβ-mediated synaptotoxicity is associated with decreased NMDARs and AMPARs-dependent excitatory synaptic transmission, decreased surface receptor levels, and spine loss. These changes in the glutamatergic function may eventually lead to synaptic depression, alterations in synaptic networks, and cognitive deficits associated with the progression of AD (Palop & Mucke, 2010).

(a) *NMDARs* are tetramers composed of two GluN1 (formerly known as NR1) subunits and two GluN2 subunits (GluN2A–GluN2D), and less commonly, the GluN3 subunit. NMDARs are ligand-gated ion channels activated by a selective pharmacological agonist called NMDA. NMDARs require the co-agonist glycine for full activation. A distinctive feature of NMDAR activation is the requirement for strong postsynaptic depolarization. Activation requires both glutamate/glycine binding and strong postsynaptic membrane depolarization to remove an internal Mg^{2+} from blocking the channel pore. NMDARs are selectively permeable to Ca^{2+} ions, which activate numerous signaling molecules including the protein kinases and protein phosphatases required for LTP and long-term depression (LTD) (Cull-Candy et al., 2001; Rebola et al., 2010).

Surface expression and channel function of NMDARs is modulated by the Src family kinases such as Fyn (Nakazawa et al., 2001; Nakazawa et al., 2006; Roche et al., 2001). Fyn phosphorylates the GluN2B subunit at a conserved motif, leading to exocytosis of the GluN1/GluN2B receptor complex. STEP opposes NMDAR surface expression by two parallel pathways: it inactivates Fyn and dephosphorylates the Tyr^{1472} of the GluN2B subunit (Braithwaite et al., 2006a; Nguyen et al., 2002; Pelkey et al., 2002; Snyder et al., 2005). Dephosphorylated Tyr^{1472} of GluN2B is a docking site for adaptor protein AP-2 and promotes the internalization of NMDA receptor by a clathrin-mediated endocytic pathway (Lavezzari et al., Roche, 2003).

Dysregulation of NMDAR function and trafficking is involved in several neuropsychiatric disorders including AD (Lau & Zukin, 2007). STEP represents one mechanism by which Aβ regulates NMDAR trafficking (Selkoe, 2008; Venkitaramani et al., 2007). Aβ binds to α7 nAChRs with high affinity and activates calcineurin (PP2B), which leads to STEP dephosphorylation within the KIM domain. Activated STEP dephosphorylates GluN2B, leading to the internalization of surface NMDARs. Application of synthetic oligomeric Aβ peptides or Aβ oligomers (derived from 7PA2

conditioned medium) to primary cortical neurons or cortical slices leads to STEP activation (Kurup et al., 2010a; Snyder et al., 2005). Reduced STEP phosphorylation is associated with decreased surface of GluN1 and GluN2B complexes. In Aβ overexpressing AD mouse models, STEP phosphorylation is significantly decreased at its KIM domain as determined by a phospho-specific antibody against STEP ser^{221} (Kurup et al., 2010a; Snyder et al., 2005). The surface expression of NMDARs is significantly elevated in STEP KO mice. Cortical cultures derived from STEP KO mice are insensitive to the affects of Aβ in mediating NMDAR receptor internalization, suggesting a critical role of STEP in regulation of NMDAR trafficking by Aβ (Kurup et al., 2010b).

(b) *AMPARs* are ligand-gated ion channels composed of the heterooligomeric subunits GluA1 to GluA4. AMPARs are permeable to cations such as Na^+ and K^+ and to a lesser extent Ca^{2+}. The presence of the GluA2 subunit in the channel makes it less permeable to calcium ions. AMPARs mediate fast synaptic transmission leading to depolarization of postsynaptic membranes and the removal of the Mg^{2+} block from NMDARs. AMPARs thus play an important role in synaptic plasticity and long-term memory (Santos, et al., 2009; Traynelis et al., 2010).

LTP and LTD are synaptic plasticity events, which are suggested to play a role in the regulation of synaptic strength. Trafficking of AMPARs play a vital role in LTP and LTD, and is regulated by different kinases and phosphatases (Anggono & Huganir, 2012). Recent studies show that tyrosine phosphatases are implicated in LTD (Moult et al., 2006). STEP is locally translated at the synapse during mGluR-dependent LTD and regulates AMPAR trafficking. Activation of mGluRs by agonist DHPG (S-3,5-dihydroxyphenylglycine) is correlated with increased STEP translation, decreased tyrosine phosphorylation of GluA2 subunit and internalization of AMPAR subunits form neuronal surface (Zhang et al., 2008). DHPG-induced AMPAR endocytosis and GluA2 dephosphorylation in hippocampal cultures and slices are blocked by a substrate trapping dominant negative STEP protein [TAT-STEP (CS)]. In addition, STEP KO cultures fail to show AMPAR internalization upon stimulation with DHPG, but are rescued by the addition of WT STEP protein (TAT-STEP WT) suggesting the role of STEP activity in AMPA receptor internalization (Zhang et al., 2008). These results suggest STEP activity is required to regulate AMPAR trafficking. The identity of the tyrosine residue(s) in GluA2 that are dephosphorylated by STEP is not known. AMPAR trafficking is modulated by Aβ. Adding Aβ to cortical cultures or slices causes synaptic depression and is associated with the loss of dendritic spines and the removal of AMPARs from the membrane (Almeida et al., 2005; Hsieh et al., 2006; Parameshwaran et al., 2007). Recent studies shed light on the role of STEP in Aβ-mediated endocytosis of AMPARs. $STEP_{61}$ levels and activity are

increased when Aβ is added to cortical cultures or slices and is associated with decreases in surface AMPAR subunits GluA1/ GluA2, as well as NMDAR subunits GluN1/GluN2B (Zhang et al., 2011). This effect is specific to Aβ oligomers but not monomers. Adding Aβ oligomers leads to STEP activation by dephosphorylation of the regulatory serine residue within the KIM domain. The catalytic activity of immunoprecipitated STEP has been analyzed by *in vitro* phosphatase assays that use a phospho-substrate corresponding to the GluA2 C-terminal region. Together, these results indicate that STEP activation contributes to the Aβ-mediated endocytosis of both NMDARs and AMPARs (Kurup et al., 2010b; Zhang et al., 2011).

D. Regulation of STEP by Beta Amyloid

Aβ peptide is derived from amyloid precursor protein (APP) by the sequential action of β and γ secretases (Turner, et al., 2003; Wolfe, 2010). The Aβ peptide that is generated by this process slowly accumulates in the brain and is thought to contribute to the pathophysiology of AD. Recent studies indicate that soluble Aβ oligomers formed in the initial stages of AD, even before amyloid plaques formation, disrupt synaptic function (Palop & Mucke, 2010). This idea is supported by studies showing that soluble Aβ oligomers inhibit LTP (Walsh et al., 2002), induce synapse loss (Lacor et al., 2007), and cause cognitive defects in animal models (Shankar et al., 2008).

STEP opposes synaptic strengthening by down regulating several enzymes involved in synaptic plasticity (Braithwaite et al., 2006b; Goelbel-Goody et al., 2012). Under normal conditions, STEP is either removed or inactivated to favor synaptic strengthening. In contrast, several neurological disorders involve STEP accumulation and overactivation. For example, Aβ activates STEP by two mechanisms: (1) dephosphorylating the KIM domain of STEP by activating PP2B (Snyder et al., 2005) and (2) blocking efficient STEP degradation by inhibiting the proteasome system (Kurup et al., 2010a). Aβ binds to the α7 nAChRs through a critical aromatic residue (Tyr^{188}) present in the agonist binding domain and activates the calcium-dependent phosphatase PP2B (Snyder et al., 2005; Tong, Arora, White, & Nichols, 2011). Activation of the PP2B/PP1 pathway dephosphorylates STEP at the KIM domain, thereby increasing the ability of STEP to interact with its substrates. As previously mentioned, PKA phosphorylation of STEP within its KIM domain inhibits the affinity of STEP for its substrates, whereas STEP dephosphorylation by PP2B/PP1 increases its affinity. PP2B-mediated STEP activation leads to increased binding and dephosphorylation of GluN2B and subsequently enhanced endocytosis of the NMDA receptors. Furthermore, Aβ-mediated NMDAR endocytosis is blocked by the α7 nAChR antagonist,

α-bungarotoxin, and by the PP2B inhibitor, cyclosporine, as well as a membrane permeable TAT-STEP (CS), which preferentially binds to STEP substrates and competes with endogenous STEP protein (Snyder et al., 2005).

Aβ also regulates STEP levels by an independent mechanism involving the UPS (Kurup et al., 2010a). Aβ inhibits proteasome activity and causes accumulation of several proteins that are normally degraded by the proteasomal pathway (Keller et al., 2000; Tseng et al., 2008). In human AD brains, decreased proteasomal activity is associated with an accumulation of ubiquitin-immunoreactive inclusion bodies (Lam et al., 2000; Mori, et al., 1987). AD mouse models and exogenous Aβ treated cultures show an accumulation of several UPS substrates, suggesting a defect in the clearance of these proteins by proteasomes (Almeida, et al., 2006; David et al., 2002; Oh et al., 2005; Qing et al., 2004). In support of this hypothesis, an increase in STEP levels is observed in cortical cultures treated with Aβ-enriched condition medium (derived from APP expressing 7PA2 cell lines) (Walsh et al., 2002). The increase in STEP levels is insensitive to translation or transcriptional inhibitors, and this effect is specific to Aβ in the conditioned medium. Immunodepletion of Aβ from conditioned medium prior to adding it to cultures blocks the increase in STEP levels. Aβ-stimulation of cortical cultures leads to dose-dependent increases in STEP levels and decreases in membrane bound NMDAR receptors. Similarly, mutant APP mouse models that express high levels of Aβ show a progressive increase in STEP levels with age that correlates with increases in Aβ species in the cortex. In this mouse model of AD, increased STEP levels are associated with decreased expression of NMDAR and AMPAR subunits in the membrane (Kurup et al., 2010a; Zhang et al., 2011). Together, these findings highlight STEP regulation by Aβ species through two parallel pathways (Fig. 2). Aβ binding to α7 nAChRs activates STEP through a PP2B/PP1-dependent dephosphorylation of STEP. Aβ also blocks STEP degradation through inhibition of the proteasome, leading to increased STEP expression. The kinetics of these events show that application of Aβ to cortical slices initially decreases phospho-STEP levels, which is followed by a gradual increase in STEP levels and then subsequent loss of surface GluN2B (Kurup et al., 2010a). Further studies with STEP KO cultures confirm the direct implication of STEP on Aβ-mediated glutamate receptor endocytosis. Treatment of wild-type cultures with Aβ-containing conditioned medium decreases the number of GluN1/GluN2B and GluA1/GluA2 subunits in the membrane as examined by surface biotinylation experiments. In contrast, Aβ-containing conditioned medium does not recapitulate the decrease in GluN1/GluN2B and GluA1/GluA2 subunits in STEP KO cultures, which clearly explained the role of STEP in mediating glutamate receptor endocytosis through Aβ (Kurup et al., 2010b; Zhang et al., 2011).

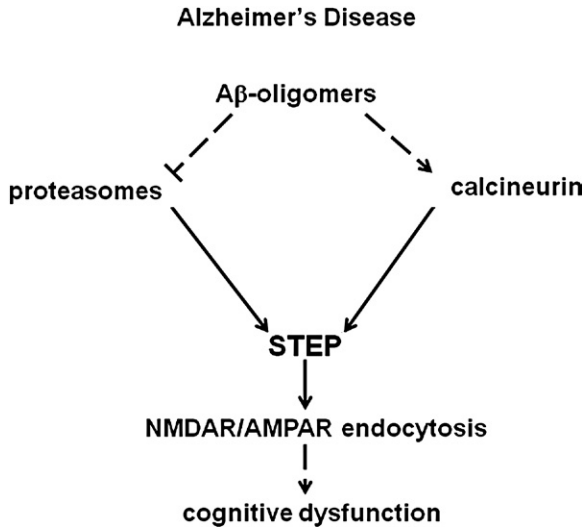

FIGURE 2 **Role of STEP in Alzheimer's disease.** In Alzheimer's disease, Aβ-oligomers activate STEP by two parallel pathways. (1) Aβ-oligomers bind to α7-nicotinic receptors and cause calcinuerin-dependent dephosphorylation of STEP at its KIM domain (2) Aβ-oligomers block the proteasome-mediated degradation of STEP and increase STEP levels. Both these events lead to increase in STEP function and increased endocytosis of NMDA and AMPA receptors causing cognitive dysfunction.

E. Transgenic AD Mouse Models

Transgenic mouse models have made important contributions to our understanding of AD (German & Eisch, 2004). These animal models carry one or more human mutant gene that is implicated in familial AD such as: APP, presenelin-1, or Tau. These models recapitulate some, but not all of the features of the disease phenotype, but have been useful for the exploration of underlying pathological mechanisms of the disease, disease progression, and to identify new therapeutic strategies (Ashe & Zahs, 2010). Although several AD mouse models have been characterized, this review focuses mainly on two widely used transgenic AD mouse models, Tg2576 and the triple transgenic mice (3×Tg-AD), for which data for STEP exist.

1. Tg2576 Mouse Model

The Tg2576 mouse model carries a mutant APP (APP_{695}SWE) found in human familial AD and produces excess Aβ. The mice show synaptic and cognitive defects in the early stages of the disease, and amyloid plaques accumulate as the disease progresses (Hsiao et al., 1996). This model has been used to test the effect of soluble Aβ in the early stages of the disease and its effects on synaptic plasticity and cognitive function. The mice show significant cognitive defects associated with reduced spine density as early as

4 months of age, decreased hippocampal neurotransmission, and decreased LTP (Jacobsen et al., 2006). These changes occur even before the apparent accumulation amyloid plaques, supporting the idea that early effects of Aβ result in synaptic perturbations.

$STEP_{61}$ levels in Tg2576 mouse brains increase progressively with age from 6 months onwards, and soluble Aβ increases in the cortex at the same time. The examination of NMDARs and AMPARs in synaptic membrane fractions of Tg2576 cortex show a significant decrease in GluN1, and GluN2B subunits of the NMDA receptor, and decreases in Tyr^{1472} phosphorylation of the GluN2B subunit (Kurup et al., 2010a). A reduction in AMPAR subunits GluA1/GluA2 is also observed in the cortical membrane fraction of Tg2576 compared to wild type (Zhang et al., 2011). The increase in $STEP_{61}$ levels is associated with decreases in STEP phosphorylation at KIM domain, suggesting that STEP is overactive and causes excessive internalization of NMDARs and AMPARs. To directly access the catalytic activity of accumulated STEP, it was immunoprecipitated from the membrane fractions of Tg2576 brain tissue and subjected to an *in vitro* phosphatase assay. STEP immunoprecipitated from Tg2576 brain shows increased phosphatase activity of a phospho-GluN2B substrate (Kurup et al., 2010a; Zhang et al., 2011).

Work in the Tg2576 AD mouse model confirms the role of STEP in glutamate receptor trafficking. This was tested in STEP knockout mice that replace the genomic STEP phosphatase domain with the neomycin gene in embryonic stem (ES) cells by homologous recombination (Venkitaramani et al., 2009). These mice are viable and show no obvious phenotypic abnormalities. Biochemical characterization of STEP KO brains show a absence of STEP expression, increased tyrosine phosphorylation of STEP substrates, and increased membrane expression of glutamate receptors, including NMDARs (Venkitaramani et al., 2009, 2011; Zhang et al., 2010) and AMPARs in synaptosomal membrane fractions (Zhang et al., 2008). The potential effect of genetically lowering STEP levels in Tg2576 mice was examined by crossing STEP KOs with Tg2576 mice, resulting in Tg2576 progeny that have high levels of Aβ but are null for STEP. Glutamate receptor levels (GluN1/GluN2B and GluA1/GluA2) were analyzed in the cortical tissue of this double transgenic mouse. As predicted, membrane expression of glutamate receptors (GluN1/GluN2B and GluA1/GluA2) was increased, suggesting that the STEP elimination is sufficient to rescue the biochemical defects in the cortex of Tg2576 mice (Kurup et al., 2010a, 2010b; Zhang et al., 2011). Aβ levels are comparable in Tg2576 and double transgenic mice in the absence of STEP, suggesting that the rescue is not due to altered Aβ metabolism or clearance.

2. *Triple-Transgenic Mouse Model*

The triple-transgenic mouse model (3×Tg-AD) of AD carries three transgenes: PS1M146V, APPswe, and tauP301L (Oddo et al., 2003a). All

three genes are implicated in human AD and the accumulation of characteristic Aβ plaques (composed of Aβ peptides) and NFTs (composed of hyperphosphorylated Tau). 3×Tg-AD mice show synaptic dysfunction and LTP defects even before the plaques and tangles are apparent. This supports the amyloid cascade hypothesis, suggesting that synaptic dysfunction caused by soluble Aβ is responsible for cognitive impairment in early stages of AD, and is independent of plaques or tangles (Oddo, et al., 003b).

The 3×Tg-AD mouse model mimics human AD in several ways, including steady-state expression of APP and the Tau transgene, which are present in the hippocampus and cortex, whereas other brain regions such as the cerebellum show the least expression (Oddo et al., 2003a). Recent studies with 3×Tg-AD (Oddo et al., 2003a) and STEP KO (Venkitaramani et al., 2009) provide direct evidence for the role of STEP in AD pathophysiology. Zhang and colleagues crossed STEP KO mice with 3×Tg-AD to produce progeny of 3×Tg-AD that are null for STEP (Zhang et al., 2010). The progeny of these double mutants were tested with behavioral tasks to assess cognitive function. At 6 months of age, 3×Tg-AD mice show significant impairment in spatial reference memory, spatial working memory, and memory tasks mediated by the hippocampus. In addition, NMDAR subunits (GluN1/GluN2B) are significantly reduced in the hippocampal synaptosomal fractions of 3×Tg-AD compared to wild type, which is associated with increases in STEP levels. Interestingly, the double mutants (3×Tg-AD-STEP KO) showed cognitive rescue in similar behavioral paradigms at 6 months of age, indicating that lowering STEP is sufficient to rescue the cognitive defects observed in the 3×Tg-AD mice.

In addition to cognitive rescue, the double mutant hippocampal synaptosomal fractions showed restored GluN1/GluN2B subunit levels, which were similar to wild-type receptor levels. Electrophysiological studies show that theta-burst LTP is significantly enhanced in double mutants compared to 3×Tg-AD. The attenuation of cognitive deficits in the double mutants occurred despite the continued elevation of Aβ and phospho-tau. This finding suggests that reducing STEP levels in the early stages of AD was beneficial (Zhang et al., 2010). Further study is needed to determine whether the cognitive rescue is restricted to early stages of AD or persists to later stages when amyloid accumulation and NFTs are increased. Nonetheless, these findings suggest that STEP is a link between the toxic effects of Aβ, synaptic dysfunction, and cognitive deficits in AD.

III. STEP Inhibitors

The integral role STEP plays in synaptic function and the striking implications for its role in AD point to this molecule as an important target for drug discovery. Identifying small molecules that inhibit STEP

activity has potential therapeutic value for the treatment of AD. Tyrosine phosphatase catalysis occurs within a highly conserved phosphatase domain. Most existing PTP inhibitors have a tyrosine phosphate-mimicking group that interacts with a highly conserved phosphate-binding loop in the catalytic center (reviewed by Blaskovich, 2009). It is also possible that small molecules that stabilize PTPs in the open inactive conformation of PTP may be useful for identifying STEP inhibitors. In the active state, the flexible WPD (Try-Pro-Asp) loop plays an important role in PTP catalysis (Barr, 2010). The WPD loop is more flexible in STEP and contains an atypical open conformation that is dominated by charged residues such as glutamine; it is located further away from the catalytic site, thereby creating a large binding pocket in the WPD loop (Eswaran et al., 2006). This binding pocket might be an interacting site for small molecules that increase the specificity for STEP compared to other PTPs. Strategies to identify STEP inhibitors are in progress; hopefully STEP inhibitors will be available in the market in near future.

IV. Conclusion

Recent advances have helped to clarify the regulation of STEP, identify its substrates, and explore its contribution to AD. STEP dephosphorylates and inactivates specific substrates including ERK1/2, p38, and Fyn that begin to explain its role in neuronal signaling. STEP down regulates membrane expression of NMDARs and AMPARs, and thereby opposes the development of synaptic strengthening. STEP KO mice show enhanced theta-burst LTP in the hippocampus and perform better in some hippocampus-dependent memory tasks. The synaptic and cognitive changes that occur in STEP KO mice are associated with increased NMDAR and AMPARs at synaptic membrane. STEP activity and function are both upregulated in AD. Increased STEP levels are found in human AD brains and in several AD mouse models. The two mechanisms that result in the upregulation of STEP activity are increased dephosphorylation as well as decreased degradation by the proteasome. Both of these events contribute to increased STEP activity and result in excessive internalization of NMDARs and AMPARs. Lowering STEP levels attenuates the biochemical and cognitive deficits observed in AD mouse models and validate STEP as a potential target for drug discovery.

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Conflict of Interest: The authors declare no conflict of interest regarding the work reported here.

Abbreviations

AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ERK	extracellular regulated kinase
NMDARs	*N*-methyl-D-aspartate receptors
PKA	protein kinase A
PTPN5	protein tyrosine phosphatase nonreceptor type five
TAT	transactivator of transcription

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Estrogen Regulation of Mitochondrial Bioenergetics: Implications for Prevention of Alzheimer's Disease

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease with a complex and progressive pathological phenotype characterized first by hypometabolism and impaired mitochondrial bioenergetics followed by pathological burden. Increasing evidence indicates an antecedent and potentially causal role of mitochondrial bioenergetic deficits and brain hypometabolism coupled with increased mitochondrial oxidative stress in AD pathogenesis. Compromised aerobic glycolysis pathway coupled with oxidative stress is first accompanied by a shift toward a ketogenic pathway that eventually progresses into fatty acid oxidation (FAO) pathways and leads to white matter degeneration and overproduction and mitochondrial accumulation of β-amyloid.

Estrogen–induced signaling pathways converge upon the mitochondria to enhance mitochondrial function and to sustain aerobic glycolysis coupled with citric acid cycle–driven oxidative phosphorylation to potentiate ATP (Adenosine triphosphate) generation. In addition to potentiated mitochondrial bioenergetics, estrogen also enhances neural survival and health through maintenance of calcium homeostasis, promotion of antioxidant defense against free radicals, efficient cholesterol trafficking, and beta amyloid clearance.

Significantly, the convergence of E2 mechanisms of action onto mitochondria is also a potential point of vulnerability when activated in diseased neurons that exacerbates degeneration through increased load on dysregulated

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calcium homeostasis. The "healthy cell bias of estrogen action" hypothesis examines the role that regulating mitochondrial function and bioenergetics play in promoting neural health and the mechanistic crossroads that lead to divergent outcomes following estrogen exposure. As the continuum of neurological health progresses from healthy to unhealthy, so too do the benefits of estrogen or hormone therapy.

I. Introduction: Alzheimer's Disease—Unlimited Cost/Limited Windows of Therapeutic Opportunity

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease and the leading cause of dementia among the aged population. It is estimated that 5.4 million people are currently living with AD in the United States, and this number is projected to at least double by the year 2050 (Alzheimer's Association, 2011). Additionally, the prevalence of AD increases exponentially with age in people aged 65 or older (Hansson et al., 2006). The majority of AD patients (about 67%) are women (Alzheimer's Association, 2011) partially because there are more women than men in the oldest segment of the population (V. W. Henderson & Brinton, 2010). Additionally, loss of ovarian hormones associated with menopause in mid-to-late life has been linked to increased risk for AD in women (Brinton, 2008b; V. W. Henderson & Brinton, 2010).

The disease is symptomatically characterized by progressive memory deficits, cognitive impairments, and personality changes, which can be attributed to deteriorating synaptic function and the subsequent loss of neurons in vulnerable regions of the brain, including the neocortex, the limbic system, and the subcortical regions (Fassbender et al., 2001). From a histopathological view, AD is characterized by senile plaques and neurofibrillary tangles (NFTs) in the medial temporal lobe and cortical areas of the brain (Hansson et al., 2006). AD has been categorized into two major forms: familial AD (FAD) and late-onset AD (LOAD; also termed sporadic AD, or SAD) with the latter being the leading cause of dementia in the elderly. FAD is an autosomal dominant disorder with onset before 65 years of age. The majority of FAD cases have been attributed to mutations in three genes: amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 *(PSEN2*) (K. Chen et al., 2007). In contrast, the complete etiology of LOAD has yet to be fully elucidated, although age has been recognized as the greatest risk factor.

Currently, no treatment exists to prevent, modify, or halt the progression of AD (Golde et al., 2011; Schneider et al., 2011). Available drugs approved by FDA only offer moderate and temporary symptom relief (Golde et al., 2011). Therapeutic developments for AD, particularly LOAD, have been largely impeded by limited understanding of disease

etiology. The prevailing "amyloid cascade" hypothesis, which was first introduced by Hardy and Higgins in 1992 and has been enriched over the past decade, emphasizes the neurotoxic characteristics of β-amyloid (Aβ) as the main contributor to disease progression. This hypothesis proposes that the deposition of Aβ initiates a cascade of events, including the formation of NFTs, prolonged inflammatory responses, increased oxidative stress and mitochondrial dysfunction, which eventually lead to cell death and dementia (Armstrong, 2011; Hardy, 2006; Hardy & Higgins, 1992; Sommer, 2002). While this "amyloid cascade" hypothesis proposes a unified etiopathogenic mechanism for both FAD and LOAD, findings from both basic research and clinical observations indicate that a far more complex mechanism underlies LOAD. Recent studies indicate that in LOAD both Aβ deposition and NFTs, rather than being the cause of the disease, may be reactive products that arise from increased vulnerability to genetic and environmental risk factors as a function of aging (Armstrong, 2011; Gibson & Shi, 2010; Pimplikar, 2009; Simon et al., 2010). Moreover, candidates that directly target amyloid pathways, either through passive immunotherapy against Aβ (Bapineuzumab) (Prins et al., 2010) or via inhibition of pathways involved in Aβ generation (Tarenflurbil, Semagacestat, or Flurizan) (Imbimbo & Giardina, 2011), failed to achieve efficacy in recent clinical trials, indicating the therapeutic limitation of amyloid-specific strategies. Increasing evidence suggests that AD, particularly LOAD, is a multifaceted disease that could at least be partially attributed to a decline in mitochondrial function and altered brain metabolic activity.

II. Role of Mitochondrial Bioenergetics in Alzheimer's Pathogenesis

A. Mitochondrial Dysfunction and β-Amyloid

The fundamental role of mitochondria in cellular bioenergetics and survival is well established (Brinton, 2008a; Magistretti, 2006; Wallace, 2005). In addition, the evidence for mitochondrial dysfunction as a pivotal factor in age-associated neurodegenerative diseases such as Alzheimer's and Parkinson's continues to mount (Brinton, 2008b; Moreira et al., 2006; Moreira et al., 2010, 2011; Mosconi, Mistur, Switalski, Brys et al., 2009; Swerdlow & Khan, 2009). Perturbations in mitochondrial function have long been observed in samples derived from clinically confirmed AD patients, including altered mitochondrial morphology, compromised enzyme complexes in the tricarboxylic acid cycle, and reduced cytochrome-c oxidase (COX) activity (Blass et al., 2000; Cardoso et al., 2004; Gibson et al., 1988; Parker, 1991; Perry et al., 1980; Sorbi et al., 1983). Later, the

"cybrid model" of AD, generated by transferring mitochondrial DNA (mtDNA) from human AD patients into cell cultures that are devoid of endogenous mtDNA (ϱ^0 cells), exhibited characteristics that recapitulated previous findings from clinical AD specimens. These findings included decreased mitochondrial mobility, increased oxidative stress, decreased COX activity, decreased mitochondrial membrane potential, and increased Aβ production, thereby providing further evidence for involvement of mitochondria and mtDNA in AD etiopathogenesis (Khan et al., 2000; Swerdlow, 2007). Increasing evidence indicates that mitochondria are direct targets of Aβ. Aβ has been demonstrated to accumulate within mitochondria and interact with a mitochondrial protein, Aβ-binding alcohol dehydrogenase (ABAD), resulting in decreased COX activity and increased oxidative stress (Lustbader et al., 2004; Reddy & Beal, 2008; Takuma et al., 2005). Further, the Aβ-induced neurotoxicity requires functional mitochondrial respiratory chain enzyme complexes (Cardoso et al., 2001) and is exacerbated in synergy with mitochondrial dysfunction in AD cybrid models (Cardoso et al., 2004).

While the neurotoxic mechanisms of Aβ converge upon mitochondria, compromised mitochondrial function, particularly a decline in mitochondrial bioenergetics and an increase in oxidative stress, propagates the degenerative process by further increasing Aβ generation. This creates a vicious cycle in which excessive Aβ accumulation and sustained mitochondrial dysfunction synergize to activate a cascade of neurodegenerative pathways (Cardoso, Santana et al., 2004; Silva et al., 2011; Swerdlow et al., 2010; Swerdlow & Khan, 2009).

B. Mitochondrial Bioenergetic Deficits in AD

Multiple levels of analysis and experimental paradigms, ranging from *in vitro* cell model systems and genomic analyses in animal models to postmortem autopsy of human brain and human brain imaging, indicate that dysfunction in glucose metabolism, bioenergetics, and mitochondrial function are consistent antecedents to development of Alzheimer's pathology (Gibson & Shi, 2010; Hauptmann et al., 2009; Nicholson et al., 2010; Yao et al., 2009). A decline in brain glucose metabolism and mitochondrial function can appear decades prior to the onset of histopathological and/or clinical features and thus may serve as a biomarker of AD risk as well as a therapeutic target (Mosconi et al., 2008; Mosconi & McHugh, 2011; Mosconi et al., 2009; Mosconi et al., 2009; Reiman et al., 2004). Studies using multiple preclinical *in vitro* and *in vivo* AD models have demonstrated a decline in mitochondrial function prior to the development of Alzheimer's pathology, including decreased mitochondrial respiration, decreased metabolic enzyme expression and activity, decreased cerebral glucose metabolism, increased oxidative stress, and increased

mitochondrial Aβ load and ABAD expression (Chou et al., 2011; Diana et al., 2011; Du et al., 2010; Hauptmann et al., 2009; Nicholson et al., 2010; Yao et al., 2009). The decline in mitochondrial function deteriorates with AD progression (Lustbader et al., 2004; Takuma et al., 2005). Consistent with basic science findings, multiple positron emission tomography (PET) studies also report antecedent abnormality in cerebral glucose utilization decades prior to the onset of AD, particularly in the hippocampal and entorhinal cortical regions (De Santi et al., 2001; Ishii et al., 1997; Mosconi et al., 2008; Mosconi et al., 2009; Reiman et al., 2004; Rosenbloom et al., 2011; Spulber et al., 2008). This distinct pattern of brain hypometabolism predicted the cognitive decline in normal aging (Mosconi et al., 2008) or the progression of patients from mild cognitive impairment (MCI) to AD (Chetelat et al., 2003) with high accuracy. Recent clinical studies revealed a significant overlap between brain regions that exhibited abnormal glucose metabolism and regions that are most vulnerable to development of AD pathology (Bero et al., 2011; Vaishnavi et al., 2010; Vlassenko et al., 2010), providing further evidence of the association between disrupted glucose metabolism and AD pathogenesis.

C. Bioenergetic Deficits and Oxidative Stress

Impairment of mitochondrial bioenergetics and oxidative phosphorylation are closely associated with increased free radical production and consequent oxidative damage. As the major source for cellular reactive oxygen species, mitochondria generate free radicals (superoxide anion, O_2^-) and hydrogen peroxide (H_2O_2) as by-products of oxidative phosphorylation (Dumont et al., 2010; Lin & Beal, 2006). It is well documented that oxidative damage to mitochondrial membranes and proteins impairs mitochondrial oxidative phosphorylation efficiency and results in increased electron leak, increased H_2O_2 levels and higher oxidative stress (Beal, 2005; Reddy & Beal, 2008). Key enzymes involved in mitochondrial bioenergetics, such as pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (αKGDH), are often the targets of oxidative modifications. This leads to deceased enzyme activity, decreased efficiency of mitochondrial electron transport, and increased production of free radicals (Park et al., 1999; Starkov et al., 2004).

Higher oxidative stress is characteristic of AD brains (Atamna & Frey, 2007; Gibson & Shi, 2010): in AD patients, significant increases in lipid peroxides, 8-oxoguanine, and oxidized amino acids, have been identified in vulnerable brain regions (Nunomura et al., 2004; Reddy, 2006). In preclinical AD animal models, increased generation of H_2O_2 and elevated oxidative damage to cellular components has also been shown to precede the development of AD pathology (Nunomura et al., 2009; Pratico et al., 2001; Rhein et al., 2009; Trushina & McMurray, 2007; Wang et al., 2005; Yao

et al., 2009). Interestingly, an increase in oxidative stress has been demonstrated to increase Aβ production *in vitro* and *in vivo* (Moreira et al., 2007; Nunomura et al., 2001; Zhang et al., 2007).

D. Alternative Fuel Sources and White Matter Degeneration in AD

In parallel to the decline in brain glucose metabolism, white matter hyperintensities are also an early hallmark of AD (Kuczynski et al., 2010; Zhang et al., 2007). Defined as an alteration in white matter integrity, these hyperintensities are first observed in the cingulum bundle, uncinate fasciculus, and superior longitudinal fasciculus of MCI patients (O'Dwyer et al., 2011). These regions are integral structures in the brain's default mode network, a system that is active when an individual is not engaged in goal-oriented activities or is at a resting state while awake. In addition to a loss in white matter integrity, patients with MCI have characteristic hypometabolism of the prefrontal and posterior cingulate cortices, and also major components of the default mode network (O'Dwyer et al., 2011; Villain et al., 2010; Vlassenko et al., 2010). Interestingly, the connectivity between these cortices is provided by the superior longitudinal fasiculus, a region where hyperintensity positively correlates with the observed hypometabolism (Kuczynski et al., 2010). This loss in white matter integrity could be a direct result of the bioenergetic shift in these two cortices, indicating a switch from the use of ketone bodies supplied from the peripheral ketogenic organ, the liver, to ketone bodies resulting from local myelin breakdown via fatty acid oxidation (FAO) by astroglia (Morris, 2005) (Fig. 1). Alternately, lesions in white matter integrity may be caused by inadequate lipid synthesis due to competition between consumption of ketones/acetyl-CoA for bioenergetics and lipid synthesis (Morris, 2005).

Considering the role of the cingulum bundle in connecting the hippocampal formation to both the prefrontal cortex and the posterior cingulate cortex, the degeneration of this white matter tract in addition to the hypometabolism of the prefrontal and posterior cingulate cortices results in the early atrophy of the hippocampus (Risacher et al., 2009; Whitwell et al., 2007) as well as impaired memory symptomatic of AD (Villain et al., 2010).

The default mode network's heavy reliance on glucose to perform aerobic glycolysis makes synaptic transmission especially susceptible to bioenergetic deficits (Vaishnavi et al., 2010; Vlassenko et al., 2010). Recently, it has been found that amyloid beta deposition and abnormal aerobic glycolysis are present in AD in a strikingly similar pattern, specifically in the default mode network (Vlassenko et al., 2010). Mitochondrial dysfunction results in a series of changes that contribute to Aβ

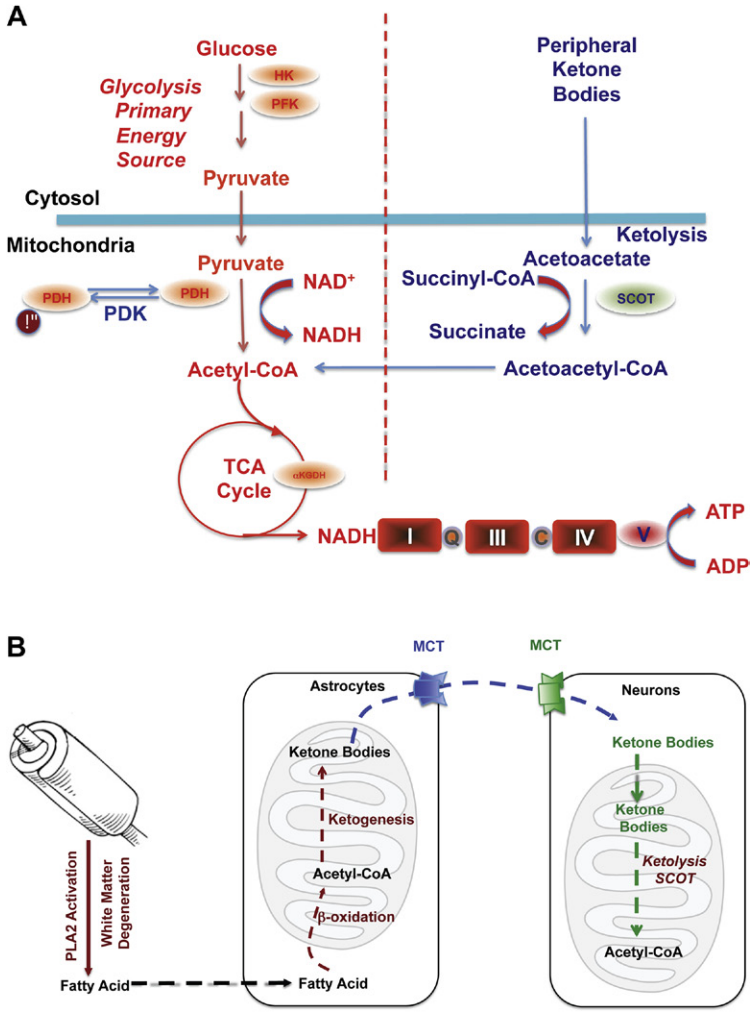

FIGURE I **Bioenergetic substrate and catalytic compensatory adaptations to sustain metabolic demand of the brain.** (A) Compensatory bioenergetic adaptation I: Glucose, the primary fuel source of brain metabolism, is converted via glycolysis to pyruvate which is further converted into acetyl-CoA to initiate and sustain the TCA cycle. Under metabolically challenging conditions (i.e., starvation, aging, and neurodegeneration) neurons can utilize peripheral ketone bodies (β-hydroxybutyrate and acetoacetate generated by the liver) through ketolysis to generate acetyl-CoA. (B) Compensatory bioenergetic adaptation II: local consumption of white matter for bioenergetics. With disease progression, peripheral ketone bodies are exhausted and the brain has to consume local white matter for energy production. Degradation of white matter via activation of PLA2 generates fatty acids that are further metabolized into acetyl-CoA through β-oxidation in the astrocytes. Acetyl-CoA is further converted into ketone bodies and transported into neurons by monocarboxylate transporters (MCTs) where ketone bodies are converted back into acetyl-CoA by SCOT and other important enzymes in ketolysis and further utilized toward ATP generation. For color version of this figure, the reader is referred to the online version of this book.

accumulation in mitochondria, including impaired oxidative phosphorylation, uncoupled electron transport chain, compromised ATP synthase, and COX inhibition (Manczak et al., 2006; Readnower et al., 2011; Young & Bennett, 2010). The fact that white matter degeneration is also selectively localized to the default mode network converges on a mechanistic pathway that links Aβ localization and activation of phospholipase A2 (PLA2). PLA2 subsequently activates sphingomyelinase, which in turn breaks down the myelin sheath to generate fatty acids that can be used in ketogenic energy production. The region-specific association between white matter degeneration, brain hypometabolism and Aβ accumulation provides compelling evidence in support of a bioenergetic mechanism that unifies both compromised glucose metabolism and white matter catabolism in AD pathogenesis.

In parallel with the decline in glucose metabolism in AD, there is a generalized shift away from glucose-derived energy production, which is associated with a decrease in the expression of glycolytic enzymes coupled to a decrease in the activity of the PDH complex (Blass et al., 2000). Alterations in the brain metabolic profile in AD are further evidenced by concomitant activation of compensatory pathways that promote the usage of alternative substrates, such as ketone bodies, to compensate the decline in glucose-driven ATP generation. We have previously reported that in the female 3xTgAD mouse model, prepathological decreases in PDH expression and mitochondrial bioenergetics were paralleled by increased expression of succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT) and hydroxyacyl-Coenzyme A dehydrogenase (HADHA) at a young age (3 months). HADHA is a subunit of the mitochondrial trifunctional protein, which catalyzes the last three steps of mitochondrial β-oxidation of long chain fatty acids to generate acetyl-CoA, whereas SCOT is the key enzyme that converts ketone bodies into acetyl-CoA. The increase in HADHA and SCOT expression indicates early activation of ketolytic and/or FAO pathways to compensate for compromised PDH capacity, and to provide alternative sources of acetyl-CoA, to sustain ATP generation (Yao et al., 2010). Consistent with these mechanistic analyses, clinical observations have also reported a substrate switch that parallels AD progression. While there is a 100:0 ratio of glucose to other substrates utilization in young controls, there is a 2:1 ratio in AD patients compared to a ratio of 29:1 in healthy elderly controls (Hoyer et al., 1991).

E. A Bioenergetic-Centric Hypothesis of AD

We have discussed four different but important pathogenic factors of AD, including decreased mitochondrial bioenergetics, increased oxidative stress, compromised white matter, and elevated Aβ generation. While each individual aspect focuses on a specific perspective of AD pathogenesis, the

unique temporal-spatial association between these components indicates a common mitochondrial-centric mechanism that unifies these aspects into a bioenergetic compensatory network. In healthy aging, the brain exhibits a glucose-driven metabolic phenotype. The energy–redox axis is tightly coupled and physiological concentrations of H_2O_2 are maintained by the coordinated activity of endogenous antioxidant systems. In contrast, in prodromal AD brains, glucose metabolism is compromised early in the disease process and creates a bioenergetic crisis, switching the brain from efficient glucose-driven energy production to less efficient ketone body-driven energy production. The compromised bioenergetic state is accompanied and further exacerbated by elevated oxidative stress, which is associated with increased expression of enzymes involved in ketogenesis and FAO, such as SCOT and HADHA, as well as mitochondrial Aβ accumulation (Young & Bennett, 2010). Further aiding this switch toward inefficiency is the elevated H_2O_2 production that results from decreased mitochondrial efficiency and increased oxidative stress. H_2O_2 leads to activation of PLA2, which degrades the myelin sheath so that it may be used as an additional source of fatty acids in ketogenesis. Consequently, the release and enrichment of free cholesterol resulting from white matter degeneration leads to impairment of the lipid-protein bilayer and contributes to hyperactivation of γ-secretase and Aβ overproduction (Burns et al., 2003; Petanceska et al., 2002; Vetrivel & Thinakaran, 2010). Cleavage of *APP* by γ-secretase leads to intraneuronal Aβ production and translocation of Aβ to mitochondria (Manczak et al., 2006; Readnower et al., 2011; Young & Bennett, 2010).

While we posit a stepwise progression of bioenergetic compensatory adaptations, it is more likely that there is a vicious cycle of exacerbating interactions. Mitochondrial accumulation of Aβ would exacerbate the bioenergetic deficits by contributing to decline in the energy-transducing efficiency. Mitochondrial accumulation of Aβ would also induce an increase in ABAD expression, perpetuating the activation of the FAO pathway and the degeneration of myelin, thereby propagating the transition of brain metabolism into a ketogenic/FAO phenotype.

III. Estrogen Action in the Brain-Convergence upon Mitochondrial Bioenergetics and Brain Metabolism

A. Estrogen-Induced Activation of Signaling Pathways: Convergence upon Mitochondria

Our investigation of estrogen regulation of mitochondrial function was stimulated by our findings that 17β-estradiol (E_2) prevented dysregulation of Ca^{2+} homeostasis by increasing mitochondrial sequestration of Ca^{2+}

while simultaneously sustaining mitochondrial respiration (Morrison et al., 2006; Nilsen & Brinton, 2003, 2004). Further, we serendipitously observed years earlier that estrogens increased ATP generation in healthy hippocampal neurons and sustained ATP generation in hippocampal neurons following exposure to $A\beta_{1-42}$ (Brinton et al., 2000). More recently, we demonstrated that *in vitro*, E2 increased maximal mitochondrial respiration in neurons and basal and maximal respiration in glia (Yao et al., 2011). E_2 pretreatment protected against inhibitors of mitochondrial electron transport chain in cultured primary neurons (Yao et al., 2011). In addition, in mice ovariectomy (OVX)-induced loss of estrogen led to significant deficits in mitochondrial bioenergetics and accumulation of mitochondrial Aβ, whereas E2 treatment initiated at time of OVX prevented the OVX-induced deficits (Yao, Irwin et al., 2011). These findings coupled with our increasing awareness that estrogen-induced signaling pathways converged upon the mitochondria (Mannella & Brinton, 2006; Nilsen & Brinton, 2003, 2004; Nilsen et al., 2006), led us to the directly investigate mitochondria as a pivotal convergence point of estrogen action in neurons (Fig. 2).

In neurons and brain, 17β-estradiol (E2) activates a system of signaling cascades, including mitogen-activated protein kinase (MAPK) (Arevalo et al., 2011; Nilsen & Brinton, 2003; Singh, Setalo, Guan, Frail, & Toran-Allerand, 2000), phosphatidylinositol-3-kinase (PI3K) (Brinton, 2008a; Cheskis et al., 2008; Spencer-Segal et al., 2011), G protein regulated signaling, c-fos, protein kinase C (PKC) (Cordey et al., 2003), and Ca^{2+} influx (T. W. Wu et al., 2005). Each of the pathways has been associated with E2 regulation of neuronal function and survival. Further, of these E2-inducible signaling pathways, PI3K has the potential for simultaneously activating the MAPK, PKC, Ca^{2+} influx, and Akt signaling pathways (Mannella & Brinton, 2006; Simoncini et al., 2000). The outcome of activating these pathways is the coordinated neuroprotective responses that involve immediate, intermediate, and long-term responses. Immediate responses involve PKC mediated phosphorylation events that rapidly open L-type calcium channels to active the Src/ERK/CREB signaling pathway. In parallel, activation of the PI3K pathway leads to phosphorylation of Akt to inactivate proapoptotic proteins (Mannella & Brinton, 2006). Intermediate responses are characterized by translocation of Ca^{2+} pERK and pAKT to the nucleus and activation of the transcription factor CREB.

B. Estrogen Regulation of Glucose Metabolism

Earlier work from the Simpkins group demonstrated that E_2 increased expression of glucose transporter subunits and increased glucose transport in blood–brain barrier endothelium (Shi & Simpkins, 1997). Later work

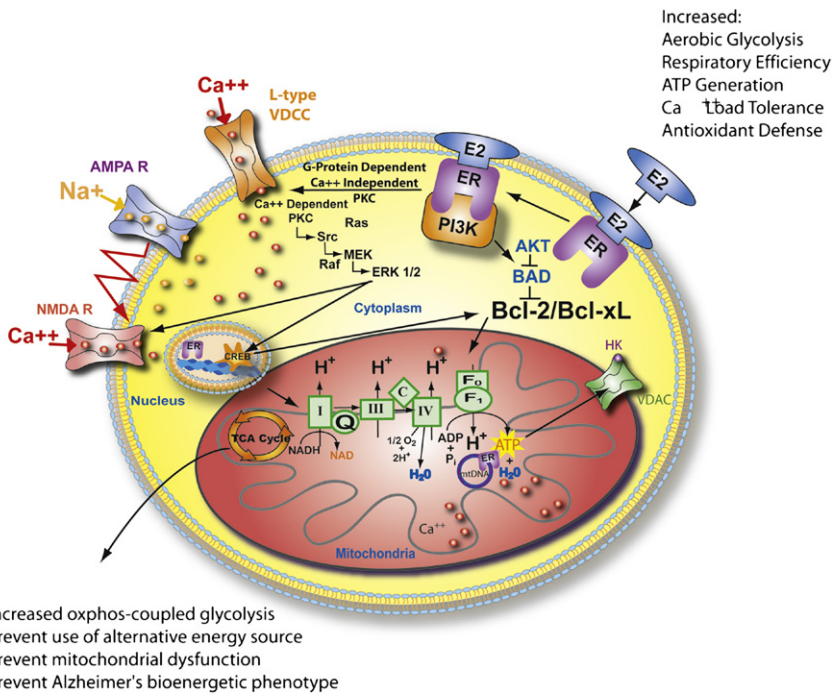

FIGURE 2 **Estrogen mechanisms of action converge upon the mitochondria.** Estrogen (17β-estradiol, E_2) binding to a membrane associated estrogen receptor (ER) undergoes a protein–protein interaction with the regulatory subunit of PI3K, p85, to activate the divergent but coordinated activation of the Akt and MAPk signaling cascades. These E_2-induced signaling pathways in hippocampal and cortical neurons converge upon the mitochondria to enhance glucose uptake and metabolism, aerobic glycolysis, pyruvate dehydrogenase to couple aerobic glycolysis to acetyl-CoA production and tricarboxylic acid cycle (TCA)-coupled oxidative phosphorylation and ATP generation. In parallel, E_2 increases antioxidant defense and antiapoptotic mechanisms. Estrogen receptors at the membrane, in mitochondria, and within the nucleus are well positioned to regulate coordinated mitochondrial and nuclear gene expression required for optimal bioenergetics. Enhancing and sustaining glycolysis, aerobic metabolism, and mitochondrial function would be predicted to prevent the shift to alternative fuel sources and the hypometabolism characteristic of Alzheimer's disease. Figure modified from (Morrison, et al., 2006). For color version of this figure, the reader is referred to the online version of this book.

by Bondy and colleagues confirmed E_2 regulation of glucose transporter proteins and that regulation of glucose transporters occurs in neurons in the nonhuman primate brain (Cheng et al., 2001). In the frontal cortex of ovariectomized nonhuman primates, E_2 treatment induced two- to four-fold increases in glucose transporter proteins Glut3 and Glut4 mRNA and protein (Cheng et al., 2001). Analysis of cellular localization indicated that E_2-induced a marked rise in neuronal Glut1 mRNA levels with no

appreciable effect on vascular Glut1 gene expression. Collectively, these data indicate that E_2 regulate metabolic functions sustaining the energetic demands of neuronal activation (Bishop & Simpkins, 1995; Nilsen & Brinton, 2003, 2004; Nilsen et al., 2006; Simpkins & Dykens, 2008; Simpkins et al., 2005).

In addition to facilitating glucose transport into the brain and into neural cells, E_2 simultaneously promotes aerobic glycolysis. Evidence derived from rat brain indicates that, *in vivo*, E_2 significantly increased glycolytic enzyme activity of hexokinase (soluble and membrane-bound), phosphofructokinase and pyruvate kinase within 4 h (Kostanyan & Nazaryan, 1992). The neuroprotective effect of E_2 is mediated by the coordinated and near simultaneous activation of both the MAPK and Akt signaling pathways through activation of PI3K in hippocampal neurons (Mannella & Brinton, 2006) (Fig. 2). Remarkably, the anti-apoptotic effect of Akt is dependent upon hexokinase association with the voltage-dependent anion channel (VDAC) of mitochondria (Gottlob et al., 2001). Hexokinases are known to bind to VDAC to directly couple intramitochondrial ATP synthesis to glucose metabolism (Miyamoto et al., 2008). Moreover, of the four hexokinase isoforms, only HKI and HKII are known to associate with mitochondria where they associate with the mitochondrial outer membrane and bind to VDAC (Gottlob et al., 2001). While it is known that E2 activates Akt (Mannella & Brinton, 2006; Singh, 2001; Znamensky et al., 2003) and increases HKII activity (Kostanyan & Nazaryan, 1992), it remains to be determined whether E_2 is promoting the association of HKII and VDAC in neural cells.

Functional impact of estrogen-induced glucose transporter protein would require a concomitant change in factors regulating glucose metabolism which in turn suggests a role for insulin or its brain homologue insulin growth factor-1 (IGF1) and its cognate receptor, IGF-1R. Bondy and colleagues found that IGF-1R mRNA was concentrated in cortical neurons in a distribution similar to Glut3 and Glut4 (Cheng et al., 2001). In the nonhuman primate frontal cortex, E2-treated animals showed a significant increase in IGF1 mRNA without a concomitant rise IGF1 receptor mRNA (Cheng, Cohen, Tseng et al., 2001). These investigators went on to elucidate the molecular mechanisms whereby IGF1 regulated neuronal metabolism by demonstrating that the active, phosphorylated form of Akt/PKB was selectively co-localized with the "insulin-sensitive" glucose transporter, Glut4, in IGF1-expressing neurons. Akt is a major target of insulin signaling in the regulation of glucose transport via the facilitative glucose transporter (Glut4) and glycogen synthesis in peripheral tissues. In parallel to these studies of glucose transport and metabolism, Garcia-Segura and colleagues have for many years demonstrated the synergistic coupling between ERs and IGF-1R (Arevalo et al., 2011; Cardona-Gomez et al., 2002; Garcia-Segura et al., 2010; Garcia-Segura

et al., 2000; Mendez & Garcia-Segura et al., 2006; Mendez et al., 2006; Spencer-Segal et al., 2011). Results of their analyses provide substantial evidence for the role of IGF-1, PI3K to Akt signaling pathway and ER in estrogen-inducible neuroprotection (Cardona-Gomez et al., 2002; Garcia-Segura et al., 2000; Mendez et al., 2003). Findings of the neuroprotective actions of the synergy between the ER and insulin/IGF-1 signaling cascades are particularly relevant to prevention of neurodegenerative diseases. In fact, in AD patients, compromised brain insulin regulation in brain regions that are vulnerable to AD pathology, including decreased expression of both insulin and insulin receptors, and impaired insulin signaling pathways, have been suggested to at least partially account for the cognitive deficits associated with this disease (Schioth et al., 2011; W. Q. Zhao et al., 2008). Torres-Aleman and coworkers have demonstrated that low circulating IGF-1 in brain is associated with greater accumulation of beta amyloid whereas Aβ burden can be reduced by increasing serum IGF-I (Carro et al., 2002). The inverse relationship between serum IGF-I and brain Aβ levels reflects the ability of IGF-I to induce clearance of β amyloid from brain, likely by enhancing transport of Aβ carrier proteins such as albumin and transthyretin into the brain (Carro et al., 2002).

C. Estrogen Regulation of Mitochondrial Function: Bioenergetic Survival for the Brain

Estrogen-mediated up-regulation of glucose transport and aerobic glycolysis is further enhanced by estrogen-induced potentiation of mitochondrial bioenergetic function. We previously conducted a proteomic analysis of brain mitochondria from female rats treated with E_2. Mitochondria, by some estimates, contain up to 1500 proteins (Lopez et al., 2007), a number that is amenable to examination by two-dimensional gel electrophoresis coupled with LC-MS/MS protein identification. Results of our proteomic analyses indicated that of the 499 detected proteins, 66 proteins had a twofold or greater change in expression (Nilsen, et al., 2007). Of these, 28 proteins were increased in expression following E_2 treatment, whereas 38 proteins decreased in expression relative to control. E_2 regulated protein expression and activity of key metabolic enzymes including PDH, aconitase, and ATP-synthase (Nilsen et al., 2007). Overall, E_2-induced marked changes in proteins involved in cellular energetics, free radical maintenance, metabolism, stress response, and cell survival (Fig. 3).

In cellular energetics, E_2 induced twofold increases in key enzymes required for glycolysis. E_2 has been reported to increase activity of the key cytosolic glycolytic enzymes hexokinase, phosphofructokinase, and phosphoglycerate kinase in rodent brain (Kostanyan & Nazaryan, 1992). In coordination with up-regulated substrate influx from increased glycolysis,

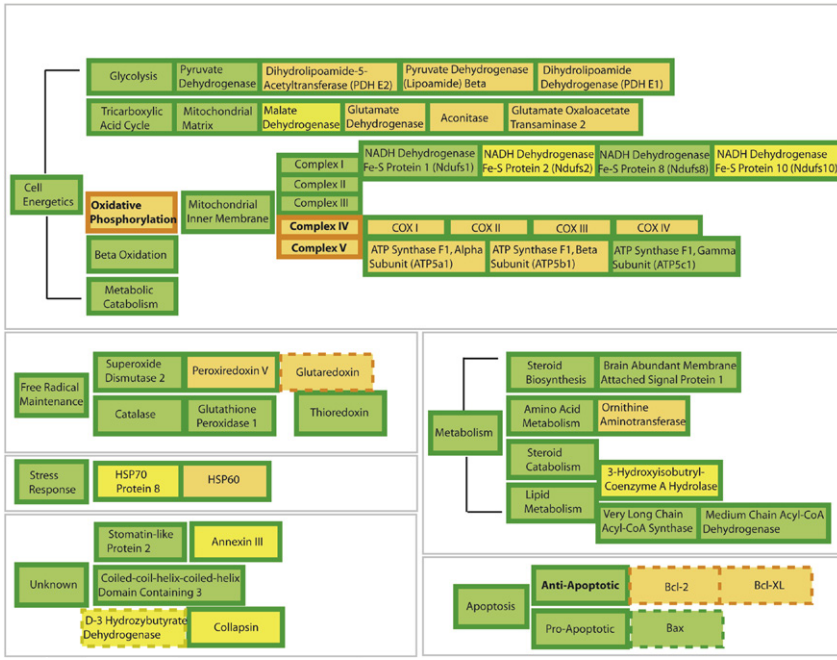

FIGURE 3 Overview of 17β-estradiol (E_2) regulation of female rat brain mitoproteome *in vivo*. Results of the functional proteomic analysis of the brain mitoproteome were combined with a bioinformatic assessment of the brain mitoproteome regulated by E_2. Proteins with known responses to E_2 were separated into functional subgroups based on common mitochondrial ontology. Orange represents upregulation and yellow represents downregulation. Filled boxes are based on results of Nilsen, Irwin et al. (Nilsen, et al., 2007). Dashed boxes are derived from published literature [reviewed in Nilsen, et al. (2007)]. Bold lettering represents altered activity. E_2 significantly increased key components of cellular energetic machinery including proteins involved in the tricarboxylic acid cycle and oxidative phosphorylation. Further, E_2 increased expression of antioxidant enzymes and antiapoptotic proteins. Collectively, the data indicates a comprehensive regulation of mitochondrial function by E_2, which increases key elements in the tricarboxylic acid cycle, pyruvate metabolism, mitochondrial oxidative phosphorylation, respiratory efficiency, and ATP generation while reducing free radical leak and oxidative damage. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

E_2 increased expression of multiple subunits of the PDH enzyme complex. PDH is a key regulatory enzyme complex linking the glycolytic metabolism to the citric acid cycle by transforming pyruvate into acetyl-CoA. In brain, PDH is further responsible for directing acetyl-CoA either to the tricarboxylic acid cycle (TCA, aka citric acid cycle) or to the acetylcholine synthesis (Holmquist et al., 2007). The mitoproteome profile induced by E_2 is reflective of enhanced glycolytic activity coupled with increased glutamatergic turnover (increased glutamate dehydrogenase and glutamate oxaloacetate transaminase-2) (Nilsen et al., 2007). Together, these findings indicate that

E_2 promotes enhanced mitochondrial utilization of glucose, the main energy source for the brain (Fig. 3).

In parallel with increased PDH expression and activity, estrogen further increased expression and activity of proteins required for oxidative phosphorylation and electron transfer, a result that was consistent with a coordinated response that optimizes glucose metabolism in brain (Nilsen et al., 2007). E_2-induced significant increased activity of Complex IV (Nilsen et al., 2007; Yao, Irwin et al., 2011)and the protein expression of its subunits I-IV (Nilsen et al., 2007), a finding consistent with previous reports (Bettini & Maggi, 1992; Stirone et al., 2005). The E2-induced increase is particularly relevant given that reduction in Complex IV is an early marker of AD (Lin & Beal, 2006). E_2 also increased expression of ATP synthase F1α and β (Nilsen et al., 2007), which is consistent with the increase in proteins required for mitochondrial respiration and with our previous report of estrogen-induced increases in ATP levels in primary neuronal cultures (Brinton et al., 2000; Yao, Irwin et al., 2011).

E_2-induced enhancement of energetic efficiency was paralleled by an increase in free radical defense systems. Many components of the mitochondrial bioenergetic network are vulnerable to oxidative stress, which can impair mitochondrial and cellular function as well as increasing apoptotic vulnerability (Lin & Beal, 2006; Yao et al., 2004). Damaged electron transport chain complexes compromise ATP synthesis and accelerate the generation of free radicals, which could cause or exacerbate neuronal degeneration (Lin & Beal, 2006; Yao et al., 2004). Free radical balance is maintained by reduction of the superoxide anion to hydrogen peroxidase by superoxide dismutases with the resulting hydrogen peroxide can then be removed by various peroxidases (Cadenas, 2004). Reduction in reactive oxygen species contributes to neuroprotection and can reduce the overall stress response. E2 treatment has been demonstrated to protect against H2O2 and arachidonic acid induced DNA damage *in vitro* (Moor et al., 2004; Tang & Subbiah, 1996). In rodent models, both short-term and long-term E2 treatments prevented the OVX-induced increase in lipid peroxides (Irwin et al., 2008; Yao, Irwin et al., 2011). Mechanistically, estrogen induces increase in the expression of a variety of antioxidant enzymes, including peroxiredoxin-V, glutaredoxin, and MnSOD (Nilsen & Brinton, 2004; Nilsen et al., 2007). In addition, we identified significant alterations in the expression of two mitochondrial heat-shock proteins (HSPs), Hsp70 and Hsp60, which are important in the correct import of nascent proteins to the mitochondrial matrix. The estrogen-induced increase in antioxidants, reduction in free radicals and substantially lower oxidative damage to mtDNA has been posited to be a major contributor to the greater longevity of females relative to males. (Borras et al., 2007; Vina, Borras, Gambini, Sastre, & Pallardo, 2005; Vina, Sastre, Pallardo, Gambini, & Borras, 2006).

Remarkably, E_2 regulation of mitochondrial function in neural tissue is closely paralleled in the vasculature (Duckles, Krause, Stirone, & Procaccio, 2006; Stirone, Duckles et al., 2005). In vascular endothelium, chronic estrogen treatment increased mitochondrial capacity for oxidative phosphorylation while simultaneously decreasing production of reactive oxygen species. In contrast to the emerging data regarding ERβ regulation of neural mitochondrial function, E_2 regulation of mitochondrial function in cerebral blood vessels is mediated by ERα (Razmara et al., 2008). Estrogen regulation of mitochondrial function in both neural and vascular tissue has functional importance for coordinated responses between neural activity and vascular integrity on the one hand and sustaining neural viability on the other.

E_2 regulated both mitochondrial and nuclear encoded gene products, requires coordinated control of mitochondrial and nuclear encoded gene transcription (Nilsen et al., 2007; Stirone, Boroujerdi, Duckles, & Krause, 2005). Neuronal estrogen receptors have been detected in mitochondria (McEwen et al., 2001; T. A. Milner et al., 2005; Stirone, Duckles et al., 2005; Yager & Chen, 2007; Yang et al., 2004). Further, both ERα and ERβ can promote neuroprotection, activate MAPK pathways, and differentially potentiate brain mitochondrial function *in vitro* and *in vivo* (Irwin et al., 2012). In addition to classical ERs, membrane sites of estrogen action (mER), which activate the PI3K/PKC/Src/MEK/ERK signaling pathway, activating CREB, have been identified as required for E_2-inducible neuroprotection (Levin, 2001; Mannella & Brinton, 2006; T.W. Wu & Brinton, 2004; X. Zhao et al., 2005). While the mechanisms whereby ERs coordinate the complex signaling pathway between the three main compartments: membrane, mitochondria, and nucleus, remain to be determined (Wagner et al., 2008), it is striking that ERs are perfectly positioned to coordinate events at the membrane with events in the mitochondria and nucleus (McEwen et al., 2001; T. A. Milner et al., 2005; T. A. Milner et al., 2008; T. A. Milner et al., 2001; Yang et al., 2004).

D. Clinical Evidence of Estrogen Regulation of Brain Metabolism *In Vivo*

As the evidence of estrogen-mediated enhancement in mitochondrial bioenergetics and brain metabolism continues to mount from basic science discoveries (Lopez-Grueso et al., 2010), multiple clinical observations further corroborate the critical role of estrogen in sustaining brain metabolism in human. As part of a 9-year study in the Baltimore Longitudinal Study of Aging, Resnick and colleagues conducted positron emission topography (PET) to assess regional cerebral blood flow in a small cohort of women who were estrogen therapy (ET) users versus women who were

not. Results of this analysis showed that ET users and nonusers showed significant differences in PET-regional cerebral blood flow relative activation patterns during the memory tasks. ET users showed better performance on neuropsychological tests of figural and verbal memory and on some aspects of the PET activation tests (Resnick & Henderson, 2002; Resnick et al., 1998). In a follow-up longitudinal study from the same cohort of healthy menopausal women, Maki and Resnick (Maki & Resnick, 2000) found that regional cerebral blood flow was increased in ET users relative to nonusers in the hippocampus, parahippocampal gyrus, and temporal lobe, regions that form a memory circuit and that are sensitive to preclinical AD (Maki & Resnick, 2000). Further these investigators found that the increase in regional cerebral blood flow was associated with higher scores on a battery of cognitive tests (Maki & Resnick, 2000). In a 2-year follow-up analysis, Rasgon and colleagues detected a significant decrease in metabolism of the posterior cingulate cortex among postmenopausal women at 2-year follow-up who did not receive estrogen whereas those women who were estrogen users did not exhibit significant metabolic change in the posterior cingulate (Rasgon et al., 2005). In addition, short-term use of estrogen has been demonstrated to enhance regional blood flow during cognitive tasks (Joffe et al., 2006; Shaywitz et al., 1999) and to enhance prefrontal–hippocampal as well as the amygdalar–cortical network connectivity (Ottowitz, Derro et al., 2008; Ottowitz, Siedlecki et al., 2008). Eberling and colleagues compared regional metabolism between healthy older women that are hormone users and nonhormone users and women with AD and found that hormone users exhibited the highest regional metabolic rate whereas AD patients exhibited the lowest metabolic rate with nonhormone users exhibit intermediate profile (Eberling et al., 2000). The same group in a follow-up study further identified that compared to the nonhormone users hormone users exhibited higher metabolic rate in the inferior frontal cortex and temporal cortex (Eberling et al., 2004). These findings that estrogen use may preserve regional cerebral metabolism and protect against metabolic decline in postmenopausal women, especially in posterior cingulate and prefrontal cortex, is particularly important given that metabolism in this region of the brain decline in the earliest stages of AD (Liang et al., 2008; Rasgon et al., 2005).

In fact, multiple clinical and epidemiological analyses clearly indicate that hormone therapy (HT) prior to or at the menopause transition is associated with enhanced memory and hippocampal function (Maki et al., 2011) and can reduce the risk of AD in postmenopausal women (Berent-Spillson et al., 2010; V. W. Henderson & Brinton, 2010) whereas women not receiving HT following surgically induced menopause are at increased risk for neurodegenerative diseases, including AD and Parkinsonism (Rocca et al., 2007; Rocca et al., 2010).

IV. Healthy Cell Bias of Estrogen Action: Consolidation of Clinical Observations and Basic Mechanistic Discoveries

Clinically, the impact of hormone interventions and the association with risk of AD has been fraught with controversy. However, this state of controversy is diminishing as a clearer understanding of the neurobiological mechanisms underlying the disparities in response to hormone therapies.

A. Prevention versus Treatment Paradigm of Estrogen Intervention

As discussed earlier, decades of basic science investigation of estrogen action in brain and subsequent observational and clinical trials indicated the benefit of estrogen-based therapies (Brinton, 2005, 2008a, 2008b; V. W. Henderson & Brinton, 2010; Morrison et al., 2006; Singh et al., 2008; Wise, 2006; Yao, Chen et al., 2011; Yao, Irwin et al., 2011). Embedded among these reports were suggestions that the beneficial effects of estrogen were conditional (Brinton, 2008a, 2008b; S. Chen, Nilsen, & Brinton, 2006; Nilsen & Brinton, 2002; Resnick & Henderson, 2002; Sherwin & Henry, 2008; Sohrabji, 2005; K Yaffe, 2003; Zandi et al., 2002). Results of the widely publicized Women's Health Initiative Memory Study (WHIMS) clinical trial drew substantial attention to how conditional ET and HT can be (Shumaker et al., 2004; Shumaker et al., 2003). Analysis of the model systems used across the basic to clinical research continuum separate into two broad classes: those that use prevention interventions in healthy organisms and those that use hormone interventions in organisms with compromised neurological function (Brinton, 2005, 2008a, 2008b). Basic science analyses that led to elucidation of the neurotrophic and neuroprotective effects of estrogen and the underlying mechanisms of action typically used a prevention experimental paradigm (Brinton, 2005, 2008a, 2008b; Yao, Irwin et al., 2011). The prevention paradigm relies on healthy neurons/brains/animals/humans as the starting foundation followed by exposure to estrogen/hormone followed by exposure to neurodegenerative insult. The prevention paradigm of basic science analyses parallels the human studies of Sherwin and colleagues who investigated the cognitive impact of ET in women with surgical or pharmacological-induced menopause (Sherwin, 2009, 2011; Sherwin & McGill, 2003). Observational, retrospective and prospective, studies are also consistent with the outcomes of basic science analyses (Brinton, 2005). For the most part, the epidemiological observational data indicate reduction in risk of AD in women who began ET or HT at the time of the menopause (Brinton, 2005, 2008b; V. W. Henderson & Brinton, 2010; K. Yaffe et al., 1998; Zandi et al., 2002). The comparable benefit observed

in most observational studies and basic science analyses suggest that for the most part, the data within the observational studies were derived from women with healthy neurological status.

In contrast, studies that fall within the second class, hormone intervention in women with compromised neurological function, that is, a treatment paradigm, exhibit a mixed profile (Brinton, 2005, 2008b). This was first evident in the results from the Cache County in which risk of AD varied with age of HT initiation and duration of use (Zandi et al., 2002). A woman's sex-specific increase in risk disappeared entirely with more than 10 years of treatment with most of the HT-related reduction in incidence reflecting former use. There was no effect with current hormone replacement therapy (HRT) use unless duration of treatment exceeded 10 years (Zandi et al., 2002). Efficacy of ET which observed in the early AD treatment trials which lasted 1.5–2 months (Fillit et al., 1986) was not sustained when ET for an extended period of time (V.W. Henderson et al., 2000; Mulnard et al., 2000). In a randomized double-blind clinical trial of ET in a cohort in aged women, >72 years, diagnosed with AD, ET resulted in a modest benefit of ET in the short term (2 months) and adverse progression of disease in the long term (12 months) (V.W. Henderson et al., 2000; Mulnard et al., 2000). In the WHIMS cohort of women, 65–79 years of age, with no indicators of neurological disease but with variable health status, no statistically significant increase in AD risk occurred in the ET/CEE arm of the trial (Shumaker et al., 2004). However, there was no benefit of ET and there was a clear decline in cognitive performance over time (Shumaker et al., 2004). In contrast, the combination of CEE + MPA for 5 years increased the risk of developing AD by twofold (Shumaker et al., 2003) and when the results of the ET and HT data were combined there was a twofold increase in the risk of AD (Shumaker et al., 2003). Subsequent post hoc analyses of the WHIMS data suggested that women who had reported prior hormone user had a significantly lower risk of AD disease and all-cause dementia during the WHIMS trials (Henderson et al., 2007).

B. "Healthy Cell Bias" Hypothesis of Estrogen Action in Brain and the "Critical Window" for Estrogen-Based Therapy: Underlying Mechanisms

Collectively, the data suggest that as the continuum of neurological health progresses from healthy to unhealthy so too do the benefits of ET or HT (Brinton, 2005, 2008b). If neurons are healthy at the time of estrogen exposure, their response to estrogen is beneficial for both neurological function and survival. In contrast, if neurological health is compromised, estrogen exposure over time exacerbates neurological demise. Based on the analyses reviewed herein, the hypothesis of a "healthy cell bias of estrogen action" is proposed (Fig. 4).

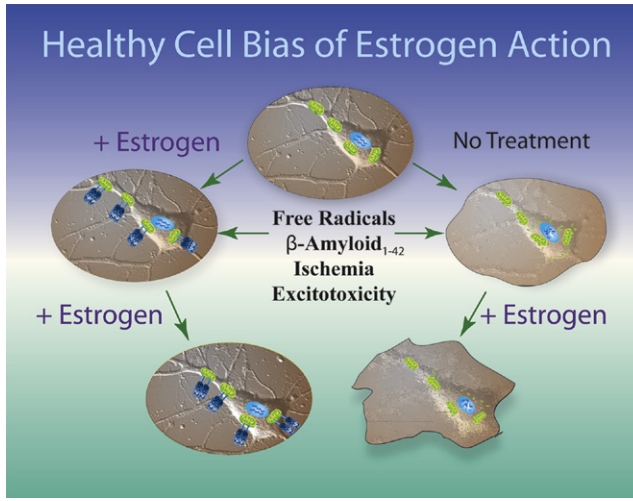

FIGURE 4 Healthy cell bias of estrogen action. Evidence from basic to clinical science indicates that neurons and women treated with estrogen prior to the exposure to neurodegenerative insult prevent neural demise. In stark contrast, basic and clinical evidence further indicate that exposure to estrogen *following* neurodegenerative insult can result in an exacerbation of neurological demise. Estrogen regulation of calcium signaling and mitochondrial function play key roles in determining the outcome of estrogen exposure. Figure modified from T. W. Wu, et al. (2005). For color version of this figure, the reader is referred to the online version of this book.

The healthy cell bias of estrogen action hypothesis predicts that ET if initiated at the time of peri-to menopause when neurological health is not yet comprised, will be of benefit as manifested as reduced risk for age-associated neurodegenerative diseases such as Alzheimer's and Parkinson's. Further, E_2 promotion of glycolysis and glycolytic coupled citric acid function, mitochondrial oxidative phosphorylation and ATP generation, antioxidant and antiapoptotic mechanisms serves as the pivotal pathway by which estrogen sustains neurological health and defense. In contrast, when activated in diseased neurons, addition of estrogen, while of modest benefit initially, an effect likely mediated by neurons not yet affected by the disease, adds to the Ca^{2+} homeostatic challenge with predictable exacerbation of the degenerative process (Chen et al., 2006). Similar to the "healthy cell bias" model in basic discoveries, the "critical window or timing hypothesis" has been proposed in clinical to interpret the disparity in outcomes between studies using the preinsult prevention paradigm and studies adopting the postinsult treatment paradigm. This hypothesis posit that the benefits and efficacy of estrogen-based HT depends stringently on the time of treatment initiation and that estrogen is most efficacious in terms of preserving cognitive function when administered prior or in the peri-to early menopausal period whereas estrogen treatment initiated years after menopause has no benefits and may even pose adverse effect (Sherwin, 2007, 2009, 2011).

Although stated differently, the healthy cell bias of estrogen action and the critical window hypothesis of clinical estrogen treatment consolidate into a unified underlying mechanism that it is the dependency upon Ca^{2+} signaling and the requirement for optimal Ca^{2+} homeostatic mechanisms that we believe is the Achilles heel of estrogen action. Through activating the PI3 kinase signaling pathway, E_2 promotes influx of Ca^{2+} via L-type Ca^{2+} channels that in turns activates the Src/ERK/CREB cascade (Fig. 2) (Mannella & Brinton, 2006; T. W. Wu et al., 2005; X. Zhao et al., 2005). Estrogen-induction of this Ca^{2+}-dependent signaling cascade leads to activation of mechanisms of learning and memory and neural defense (Brinton, 2001; Morrison et al., 2006; Woolley, 2007). Our studies of E_2 regulation of intracellular Ca^{2+} dynamics and homeostasis originated in an attempt to resolve the paradox of dual regulation of [Ca2+]i by E_2 in hippocampal neurons after nontoxic and excitotoxic glutamate exposure (Nilsen et al., 2002). Analyses of [Ca2+]i dynamics between the cytosolic and mitochondrial compartments revealed that E_2 caused an increase in mitochondrial sequestration of [Ca2+]i when neurons were exposed to excitotoxic glutamate, which was paralleled by attenuation of cytoplasmic [Ca2+]i (Nilsen & Brinton, 2003). E_2-induced attenuation was correlated with an increase in Bcl-2 expression, which could provide a mechanism by which neurons are protected against deleterious effects of increased mitochondrial [Ca^{2+}] (Murphy et al., 1996; Nilsen & Brinton, 2003). Further, the increased mitochondrial sequestration of Ca^{2+} induced by E_2 protected neurons against adverse consequences of excess cytoplasmic Ca^{2+} and subsequent dysregulation of Ca^{2+} homeostasis. Despite an increased mitochondrial Ca^{2+} load, E_2 preserved mitochondrial respiratory capacity (Nilsen & Brinton, 2003).

The above mechanistic studies were conducted in healthy neurons derived from embryonic hippocampus, we therefore sought to determine whether E_2 regulation of Ca^{2+} homeostasis extended to neurons derived from middle-aged and aged rodent hippocampus (Brewer, Reichensperger, & Brinton, 2006). Results of these analyses were both striking and consistent with earlier observations. Age-associated dysregulation of [Ca^{2+}]i homeostasis was prevented by 48 h of prior exposure to E_2 , a time frame consistent with E_2-induced Bcl-2 expression (Nilsen & Brinton, 2003; T. W. Wu et al., 2005). Embryonic neurons exhibited the greatest capacity to regulate Ca^{2+} homeostasis followed by middle-age neurons (Brewer et al., 2006). In neurons derived from aged rat hippocampus, the first peak of [Ca^{2+}]i was substantially greater than at other ages and the return to baseline Ca^{2+} rapidly dysregulated with an inability to restore [Ca^{2+}]i following the first glutamate pulse that persisted throughout the 20 pulses. E_2 pretreatment of aged neurons profoundly attenuated the peak [Ca^{2+}]i rise and delayed the age-associated dysregulation of baseline [Ca^{2+}]i, normalizing responses to those of middle-age neurons treated with E_2 (Brewer et al.,

2006). In a series of experiments designed to address controversies of ET, we conducted *in vitro* experiments designed to simulate the WHIMS trial in a dish. We hypothesized that E_2 exposure of healthy neurons in a prevention mode would promote Ca^{2+} homeostasis to prevent $A\beta_{1-42}$-induced neurodegeneration whereas E_2 exposure of degenerating neurons in a treatment mode would exacerbate $A\beta_{1-42}$-induced Ca^{2+} homeostatic dysregulation (S. Chen et al., 2006). Results of those analyses indicated that in a prevention mode of exposure, E_2 was most effective when present *prior to and during* $A\beta_{1-42}$ insult. In contrast, E_2 treatment following $A\beta_{1-42}$ exposure was ineffective in reversing Aβ-induced degeneration and exacerbated $A\beta_{1-42}$-induced cell death. We further found that low E_2 significantly prevented $A\beta_{1-42}$-induced rise in $[Ca^{2+}]i$ whereas high E_2 significantly increased $[Ca^{2+}]i$ and did not prevent $A\beta_{1-42}$-induced $[Ca^{2+}]i$ dysregulation (S. Chen et al., 2006). Therapeutic benefit resulted only from low dose E_2 exposure prior to, but not following, $A\beta_{1-42}$-induced neurodegeneration. Collectively, these data support a role of low dose E_2 in promoting Ca^{2+} homeostasis in healthy embryonic, middle-aged and aged neurons. Further, the data indicate that once dysregulation of Ca^{2+} homeostasis has occurred, as in the case of $A\beta_{1-42}$-induced Ca^{2+} dysregulation, exposure to low-dose E_2 is of no benefit and exposure to high-dose E_2 is deleterious and exacerbates neural demise. In addition to the preclinical investigations, multiple clinical studies further confirmed that the benefit of estrogen-based HT is indeed at least partially dependent on the time of initiation. Recent analyses by Whitmer and colleagues in a large clinical database revealed that use of HT in midlife may protect against cognitive impairment whereas HT initiation in late life could have deleterious effects (Whitmer et al., 2011). In a separate study, Smith and colleagues reported that early initiation of HT in menopausal women is associated with increased hippocampal and posterior cingulate cholinergic activity (Smith et al., 2011). Similarly, Gorenstein *et al* evaluated the effect of estrogen replacement therapy on verbal cognitive performance of middle-aged postmenopausal women and reported better performance of the estrogen group on digit span-forward and on the recall of the easy stimuli on the verbal-paired associates tests despite the magnitude of benefits is moderate (Gorenstein et al., 2011).

V. Clinical Implications for Biomarker Identification and Therapeutic Development for Alzheimer's Disease

Investigating mechanisms of estrogen action in parallel to identifying events antecedent to the development of Alzheimer's pathology that have mechanistic plausibility provide insights into the basis for disparities between basic science discovery and clinical outcomes. More generally, results of these investigations raise questions regarding applying preventive

strategies to treatment modalities in the clinical realm and the reliance of healthy model systems that are abruptly exposed to neurodegenerative insults that typically develop incrementally, slowly and accumulate over time in the preclinical discovery realm. This is particularly true for age-associated neurodegenerative diseases in which the normal aging brain undergoes dramatic changes that are either unrelated to or are the earliest signs of neurodegenerative vulnerability (Blalock et al., 2003; Blalock et al., 2004; Miller et al., 2008; Rowe et al., 2007; Toescu, Verkhratsky, & Landfield, 2004). Efforts to bridge these gaps in women's cognitive health are emerging and hold the promise to serve as a model for mechanistic and translational neuroscience research at the bench and the bedside (Asthana et al., in press and http://www.nia.nih.gov/ResearchInformation/ExtramuralPrograms/NeuroscienceOfAging/NNA_Conferences/BenchtoBedside.htm).

The real and perceived risks of HT remain and were amplified by results of both the WHI and WHIMS trials. It is clear that many, *but not all*, women could potentially benefit from ET or HT intervention. Biomarkers to identify women appropriate for and which type of hormone regimen remains largely undeveloped beyond the hot flash (Gleason, Dowling, Friedman, Wharton, & Asthana, 2011; Yao, Rettberg et al., 2011).

Considering the central role of mitochondrial bioenergetics and brain metabolism in AD pathogenesis and in estrogen action in the brain, it may well serve as a valid target for both biomarker development for early identification of the at AD risk population and for therapeutic development for AD prevention and treatment.

A. Development of Bioenergetic-Centric Biomarkers for AD

Recently, the clinical phases of AD have been expanded to include presymptomatic AD, during which time an individual appears cognitively normal but is beginning to exhibit some of the pathological changes of AD (Sperling et al., 2011). Defining this presymptomatic phase was particularly important for explaining why some individuals have no cognitive deficits but, upon autopsy, amyloid plaques and NFTs are present in the brain (De Meyer et al., 2010; Jack et al., 2008; Knopman et al., 2003; Mintun et al., 2006; Price & Morris, 1999). This provides further confirmation that a successful therapeutic intervention for AD will require very early identification of prodromal AD patients. Therefore, an area of concentrated focus within the Alzheimer's research community is the identification and validation of biomarkers–biospecimen or neuroimaging variables that can be used to reliably predict individuals at risk of developing AD. Development of a biomarker profile of AD would be of great benefit both to clinicians and the drug development community; clinicians so that accurate diagnoses could be made antemortem, and pharmaceuti-

cal companies so that the efficacy of new drug formulations could be tested (Williams, 2011).

The criteria for a biomarker of AD were proposed in 1998 by the Working Group on Molecular and Biochemical Markers of AD, and have since become standards for the field. The Working Group specified: "the ideal biomarker for AD should detect a fundamental feature of neuropathology and be validated in neuropathologically confirmed cases; it should have a diagnostic sensitivity >80% for detecting AD and a specificity of >80% for distinguishing other dementias; it should be reliable, reproducible, noninvasive, simple to perform, and inexpensive." The challenge of developing biomarkers that measure preclinical AD is that they must be able to discriminate between individuals who have AD pathology and those who do not, but all while the individuals are still at a cognitively intact stage so there is adequate time for prevention. As the prodromal stage of AD is known to exist decades prior to the manifestation of clinical symptoms, this would imply that preventative measures will require a method for routine screening of all patients in the age range of 50–65 years. Thus, a useful biomarker would need to be not only specific and sensitive but also cost-effective, so it would be broadly accessible.

Currently, the most thoroughly studied biomarkers of AD are the levels of three proteins measured in the cerebrospinal fluid (CSF): amyloid β_{1-42} (Aβ42), total tau protein, and p-tau, a phosphorylated form of tau protein. CSF levels of Aβ42 are decreased in AD, which is predicted to be due to the incorporation of Aβ42 into amyloid plaques (Blennow, Vanmechelen, & Hampel, 2001). CSF levels of both total tau and p-tau are increased, likely due to degenerating neurons releasing these proteins into the CSF (Jack et al., 2010). All three of these biomarkers have been validated, but changes in CSF levels of these proteins are likely occurring far downstream of the initial mitochondrial bioenergetic crisis; this implies that by the time they are measurable, the window for disease prevention may have already passed.

A recent development in the biomarker field has been the use of neuroimaging. Magnetic resonance imaging (MRI) can be used to visualize changes in brain structure that are associated with AD. Longitudinal MRI studies conducted by the Alzheimer's Disease Neuroimaging Initiative (ADNI) showed a pattern of temporal lobe atrophy that was significantly greater in patients who converted from MCI to AD than those who did not convert (Trojanowski et al., 2010). In addition, loss of hippocampal volume proved indicative of AD pathology and correlated with the APOε4 allele in FAD (Trojanowski et al., 2010). Unfortunately, by the time volumetric changes are quantifiable, substantial loss of grey and white matter has already occurred. Thus, while MRI detection of atrophy is useful as a diagnostic tool, its utility for prevention of AD is limited.

PET scanning is another neuroimaging method that has been used to study the development of AD. One type of PET imaging uses radiolabeled molecules which bind and label amyloid in the living brain. The most thoroughly studied examples of these compounds are Pittsburgh Compound B (PiB) (Klunk et al., 2004) and 18F-AV-45 (florbetapir) (Wong et al., 2010). Studies conducted using brain tissue from autopsy-confirmed AD patients show that PiB binds only to fibrillar amyloid, particularly plaques that are immunoreactive for Aβ40 or Aβ42 (Ikonomovic et al., 2008). 18F-AV-45 has also been shown to bind selectively to Aβ plaques in the postmortem AD brain (Choi et al., 2009). Unfortunately, amyloid neuroimaging techniques suffer from the same limitations as measurements of CSF Aβ42 and tby the time Aβ42 is aggregated into plaques, the pathogenesis of AD is established in brain. Additionally, PiB binding is not a 100% reliable biomarker, as there are cases of autopsy-confirmed AD that failed to show PiB labeling in the brain (Rosen et al., 2010).

Using fluorodeoxyglucose PET (FDG-PET), a significant body of research indicates that abnormalities in cerebral glucose utilization appear decades prior to the onset of clinical AD (de Leon et al., 2001; Mosconi, Mistur, Switalski, Tsui et al., 2009; Reiman et al., 2004). Further, the decrease in brain metabolism precedes the atrophy detected by MRI (De Santi et al., 2001) and predicts a decline in cognitive function (de Leon et al., 2001; Jagust et al., 2006; Mosconi et al., 2008). A decline in glucose metabolism could be simply due to decrease in brain mass; however, deficits in brain metabolism exceeded the magnitude of cortical atrophy (Ibanez et al., 1998). Based on a bioenergetic perspective of the etiology of AD, brain hypometabolism represents a response to an antecedent shift from utilizing glucose to requiring the alternative fuels of fatty acids and derived ketone bodies. Thus, hypometabolism still may be too late in the etiological cascade of events to be used as a biomarker for AD prevention, but could be applicable to identifying prodromal AD. Indeed, hypometabolism measured by FDG-PET has been identified as a "gold standard" for early-stage diagnosis of AD, although this method is hampered by high cost and relative inaccessibility of the scanning equipment.

Considering the central and antecedent role of mitochondrial bioenergetics in AD pathogenesis, a biomarker that reliably detects a shift to inefficient mitochondrial bioenergetics in the brain could provide the earliest indication that an individual is at risk for AD. Based on the Working Group recommendations that a biomarker be simple to measure and inexpensive, and our requirement that it be broadly accessible, the most desirable biomarker would be measurable in blood samples. One such marker could be plasma levels of ketone bodies. In preclinical models, for example, the 3xTgAD mouse, brain mitochondrial levels of enzymes involved in ketone body catabolism are upregulated very early in the disease process (Chou et al., 2011), suggesting a compensatory response that may be indicative of

a shift toward the use of an alternate fuel source due to ineffective glucose metabolism in brain. Elevated levels of ketone bodies in the plasma would be then expected to indicate increased ketone generation by the liver in response to the disrupted brain glucose metabolism (Fig. 1).

Mitochondrial enzyme activity also holds promise as a potential biomarker of preclinical AD. It is well established that complex IV activity is decreased in platelet mitochondria isolated from individuals with AD (Bosetti et al., 2002; Cardoso, Proenca et al., 2004). Additionally, Valla *et al* reported a decrease in platelet mitochondrial complex IV that was present in MCI patients as well as patients with AD, suggesting that changes in mitochondrial complex IV activity may be occurring early in AD pathogenesis (Valla et al., 2006). Interestingly, it was recently reported that platelet mitochondrial complex IV activity is reduced in young, cognitively normal individuals who have a maternal history of LOAD (Mosconi et al., 2011). Mitochondrial DNA is maternally inherited and codes for the proteins which make up complex IV, suggesting that some forms of LOAD may result from a maternally transmitted mitochondrial deficit. Reduced platelet mitochondrial COX activity has potential as an early marker for individuals with a maternally inherited risk of LOAD.

Activity of mitochondrial enzyme complexes has also been investigated in lymphocytes, with varying degrees of success. Some studies have found no effect of AD status on lymphocyte mitochondrial activity (Molina et al., 1997), whereas a recent study showed increased activity of mitochondrial respiratory chain complexes II and IV in lymphocytes isolated from patients with AD when compared with controls (Feldhaus et al., 2011). Another study showed that although there was no baseline difference in lymphocyte mitochondrial enzyme activities between controls and AD patients, those patients who were treated with the cholinesterase inhibitor rivastigmine showed increased activity of complexes II, III, and IV, indicating that increased mitochondrial efficiency might be associated with better disease outcome.

B. Therapeutics Targeting Mitochondria and Bioenergetics for AD Treatment and Prevention

Alzheimer's is a neurodegenerative disease with a complex and progressive pathological phenotype characterized first by hypometabolism and impaired mitochondrial bioenergetics followed by pathological burden. The progressive and multifaceted degenerative phenotype of Alzheimer's suggests that successful treatment strategies need to be equally multifaceted and stage specific. Increasing evidence indicates an antecedent and potentially causal role of mitochondrial bioenergetic deficits and brain hypometabolism coupled with increased mitochondrial oxidative stress in AD pathogenesis. Mitochondrial deficits have been demonstrated to activate a

cassette of neurotoxic events that all contribute to synaptic dysfunction, pathology development and eventually neuronal loss and cognitive impairment (Beal, 2005; Reddy & Beal, 2008). Further, deficits in mitochondrial bioenergetics and brain metabolism exhibit a stage-specific trajectory with disease progression, which was first evidenced by the decline in glucose uptake and utilization that takes place decades prior to AD onset, followed by parallel activation of pathways to use alternative fuel substrates, ketone bodies, to compensate for the decline in glucose metabolism (Yao et al., 2010; Yao et al., 2009). As disease progresses, exacerbated decline in glucose utilization and exhaustion of available ketone reservoir leads to further disturbance of mitochondrial function and activation of FAO pathway that eventually results in white matter degeneration and neuronal death observed in AD (Bartzokis et al., 2004; Brinton, 2008a; Carmichael et al., 2010; Kuczynski et al., 2010). This unique trajectory of glucose-ketone-FAO progression of brain mitochondrial metabolic alteration provides an ideal therapeutic target that is both disease modifying and stage specific (Fig. 5).

Candidates that potentiate mitochondrial bioenergetics and enhance brain glucose metabolism are expected to prevent the antecedent decline in brain glucose metabolism, promote healthy aging and therefore prevent AD.

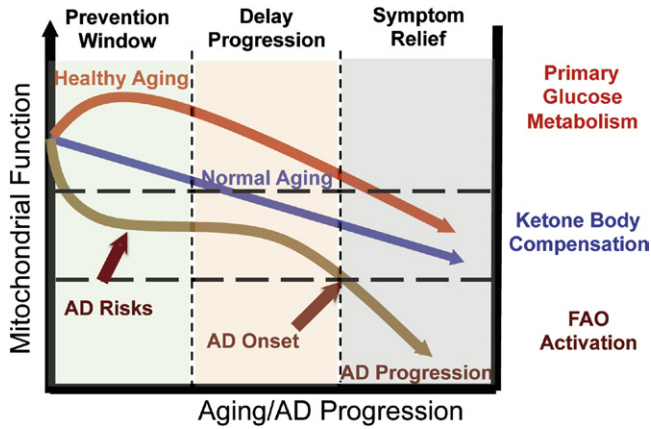

FIGURE 5 **Trajectory of mitochondrial function, substrate utilization during AD progression and therapeutic strategy.** At young age or in healthy aging, brain metabolic activity is supported by glucose, the primary fuel source, whereas in prodromal and incipient AD the antecedent decline in glucose metabolism is first paralleled by compensatory activation of ketogenic pathways, which later diminishes and progresses into local fatty acid oxidation and white matter degeneration. The prevention strategy aims to enhance the glucose-driven mitochondrial bioenergetics to promote healthy aging and prevent AD. Alternatively, in prodromal and incipient AD, sustained activation of ketogenesis provides prolonged supplement of the alternative fuel source, ketone bodies, and therefore sustains mitochondrial bioenergetic function and prevents/delays further progression of the disease. At the middle to late stage of AD, rather than modifying disease progression, treatments merely offer symptom relief. For color version of this figure, the reader is referred to the online version of this book.

Interestingly, many candidates within this category are naturally occurring herbals and small cofactors, which often are on the GRAS (generally recognized as safe) list. R-α-lipoic acid, an important cofactor for key mitochondrial metabolic enzymes, including PDH, αKGDH, and branched chain α-ketoacid dehydrogenase (BCKDH), has been demonstrated to up-regulate mitochondrial bioenergetics, promote glucose metabolism, and suppress oxidative stress due to its potent antioxidant capacity (Packer & Cadenas, 2011). Resveratrol, a redox active ingredient in grapes and wine, improves brain energy metabolism and reduces amyloid accumulation in preclinical animal models (Karuppagounder et al., 2009; Marambaud et al., 2005; O'Dwyer et al., 2011; Vingtdeux et al., 2008). Both R-α-lipoic acid and resveratrol are currently under clinical trials for their efficacy in AD prevention and treatment (Packer & Cadenas, 2011; Wollen, 2010). Other important regulators of mitochondrial metabolic activity include B-vitamins which are also cofactors of key metabolic/mitochondrial enzymes.

Another class of natural products that are of great potential for AD prevention is the isoflavones, naturally rich in soy and soy-based diets. These plant derived phytoestrogens are a class of naturally occurring polyphenolic molecules that structurally resemble the mammalian estrogen (L. Zhao & Brinton, 2007; L. Zhao et al., 2009) but have a binding preference for ERβ with weaker affinities. High intake of soy-derived phytoestrogens has been linked to the low prevalence rate of AD in Asia (L. Zhao & Brinton, 2007). Further, multiple studies demonstrated that phytoestrogens, particularly various forms of isoflavones, regulate mitochondrial function by modulating mitochondrial oxidative stress (Huang & Zhang, 2010), activating the Akt signaling pathway, promoting expression of anti-apoptotic proteins (Xing et al., 2011), and potentiating mitochondrial bioenergetic capacity (L. Zhao et al., 2009).

Due to their structural properties, many of these naturally occurring compounds can often act as free radical scavengers directly. All together, these compounds exhibit potential to promote brain glucose utilization, potentiate brain metabolic activity, and simultaneously suppress oxidative damage with relatively low toxicity, which make them ideal candidates for development of nutraceutical cocktails to promote brain metabolism during healthy aging and therefore prevent AD.

While the preventive strategy focuses heavily on the enhancement of brain glucose metabolism, the shift toward an alternative fuel source, ketone bodies, observed in both preclinical AD models and in AD patients provides a second therapeutic window that targets the specific glucose–ketone transition stage to sustain brain metabolic activity and therefore prevent or delay further exacerbation in brain bioenergetic deficits. Ketone bodies are mainly synthesized in the liver through FAO and are well documented to serve as alternative energy substrates for the heart, muscle, and brain. Ketogenic pathways have been demonstrated to exist in astrocytes (Auestad et al., 1991;

Guzman & Blazquez, 2004). Epidemiological analyses indicate a positive association between dietary intake of ketones/consumption of ketogenic diets and reduced risk for AD (S. T. Henderson, 2008; Morris, 2005). The switch from glucose as the primary fuel to the alternative of ketone bodies in the AD brain was the basis for Accera to develop Ketasyn, which is converted to ketone bodies in the liver for subsequent use by the brain. This approach capitalizes on the brain's relative inability to utilize glucose and its dependency on ketone bodies. Phase II clinical trial in AD patients and in individuals suffering from age-associated memory impairment has been completed and both groups showed improvement in memory function using the ketone body alternative fuel source (http://www.accerapharma.com).

While increasing ketone body supply provides more substrate to the brain to utilize as an alternative fuel, the therapeutic efficacy could be limited due to a diminished brain capacity to utilize ketone bodies. To address the issue of deficits in the ketogenic metabolic pathway, our group investigated the efficacy of the ketogenic modulator, 2-deoxy-D-glucose (2-DG) to increase brain capacity to utilize ketone bodies as fuel. Results of these analyses demonstrated that dietary 2-DG intake induced ketogenesis, sustained mitochondrial bioenergetics, and reduced pathology in the triple transgenic Alzheimer's (3xTgAD) mouse model (Jia Yao, 2011). Based on these clinical and preclinical findings, a combination of nutraceutical and pharmaceutical modulators that simultaneously enhance mitochondrial bioenergetics while sustaining availability and utilization of an alternative fuel substrate (ketone bodies), could prevent further decline in brain metabolism and to delay progression of AD.

VI. Conclusion

Alzheimer's disease is a complex disease with a prolonged trajectory of etiopathogenic changes in brain bioenergetics decades prior to the clinical onset of the disease. Although it remains to be clinically confirmed, the trajectory of alterations in brain metabolic profile provides the foundation upon which to develop an array of bioenergetic-centric biomarkers to predict AD risk at the preclinical stage and therefore provide the best opportunity to prevent and/or delay the onset of AD. From a therapeutic perspective, this unique trajectory of alterations in brain metabolic capacity enable a bioenergetic-centric strategy that targets disease-stage specific profile of brain metabolism for disease prevention and treatment. A combination of nutraceutical and pharmaceutical interventions that enhances glucose-driven metabolic activity and potentiate mitochondrial bioenergetic function could prevent the antecedent decline in brain glucose metabolism, promote healthy aging, and prevent AD. Alternatively, during the prodromal incipient phase of AD, sustained activation of ketogenic metabolic pathways coupled with

supplement of the alternative fuel source, ketone bodies, could sustain mitochondrial bioenergetic function to prevent or delay further progression of the disease.

In healthy brain and neural cells, estrogen coordinates activation of signaling pathways that converge upon the mitochondria to sustain aerobic glycolysis and enhance citric acid-driven oxidative phosphorylation and ATP generation. E_2-induced potentiation of aerobic glycolysis and mitochondrial glucose utilization would be predicted to prevent conversion of the brain to using alternative sources such as ketone bodies and the subsequent ketone-FAO progression. Such convergence of estrogen-induced signaling onto mitochondria is also a point of vulnerability when activated in diseased neurons, which exacerbates degeneration through increased load on dysregulated calcium homeostasis. As the continuum of neurological health progresses from healthy to unhealthy, so too do the benefits of estrogen-based HT. The diversity of estrogen-inducible outcomes requires advances in biomarkers to identify women who will benefit from vs those who should not receive estrogen therapy. Identification of early stage changes in bioenergetic capacity of brain that are preventable or reversible by estrogen therapy holds promise to prevent or reduce the risk of developing Alzheimer's disease.

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Conflict of Interest Statement: Patent pending on 2-deoxy-D-glucose formulations for prevention and treatment of Alzheimer's disease. The pending patent entitled "2-Deoxy-D-Glucose Formulations for Prevention or Treatment of Neurodegenerative Diseases" (serial number: 61/452,463) was filed on March 14, 2011 subsequent to all data collection and analysis.

Abbreviations

Aβ	amyloid beta
ABAD	Aβ-binding-alcohol-dehydrogenase
AD	Alzheimer's disease
E_2 17β	estradiol
FAD	familial AD
LOAD	late-onset Alzheimer's disease
MRI	magnetic resonance imaging
OXPHOS	oxidative phosphorylation
PET	positron emission tomography
SAD	sporadic AD

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