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## Targets for AD treatment: conflicting messages from $\gamma$ -secretase inhibitors

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### Abstract

Current evidence suggests that Alzheimer's disease (AD) is a multi-factorial disease that starts with accumulation of multiple proteins. We have previously proposed that inhibition of  $\gamma$ -secretase may impair membrane recycling causing neurodegeneration starting at synapses (Sambamurti et al., 2006). We also proposed familial AD (FAD) mutations increase A $\beta$ 42 by inhibiting  $\gamma$ -secretase. Herein, we discuss the failure of Eli Lilly's  $\gamma$ -secretase inhibitor, semagacestat, in clinical trials in the light of our hypothesis, which extends the problem beyond toxicity of A $\beta$  aggregates. We elaborate that  $\gamma$ -secretase inhibitors lead to accumulation of amyloid precursor protein (APP) C-terminal fragments (CTFs) that can later be processed by  $\gamma$ -secretase to yields bursts of A $\beta$  to facilitate aggregation. Although we do not exclude a role for toxic A $\beta$  aggregates, inhibition of  $\gamma$ -secretase can affect numerous substrates other than APP to affect multiple pathways and the combined accumulation of multiple peptides in the membrane may impair its function and turnover. Taken together, protein processing and turnover pathways play an important role in maintaining cellular homeostasis and unless we clearly see consistent disease-related increase in their levels or activity, we need to focus on preserving their function rather than inhibiting them for treatment of AD and similar diseases.

### Introduction

Alzheimer's disease (AD) is a major public health problem affecting a large fraction of the elderly population (Hebert *et al.* 2003). Unfortunately there are currently no proven therapies that delay the onset or prevent the progression of AD. However, multiple genetic, epigenetic, environmental, life style factors such as exposure to metals, smoking, high cholesterol, diabetes and elevated homocysteine have been identified as potentially valuable targets for modifying risk (Bhat 2010, Lahiri *et al.* 2008, Sambamurti *et al.* 2004). Historically, the molecular era of research on AD starts with the seminal studies that identified the sequences of the major component of the senile plaque (SP) core as the 39-42 amino acid (aa) amyloid or A4 peptide (A $\beta$ ) (Glenner & Wong 1984b, Glenner *et al.* 1984, Glenner & Wong 1984a, Masters *et al.* 1985, Wong *et al.* 1985) and neurofibrillary tangles

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(NFTs) as the microtubule associated protein, Tau (MAPT) (Baudier & Cole 1987, Bancher *et al.* 1987, Wood *et al.* 1986, Pollock *et al.* 1986, Nukina & Ihara 1986, Montejo de Garcini *et al.* 1986, Kosik *et al.* 1986, Ihara *et al.* 1986, Grundke-Iqbal *et al.* 1986b, Grundke-Iqbal *et al.* 1986a, Delacourte & Defossez 1986, Brion *et al.* 1986). Screens of cDNA libraries detected eight isoforms of the amyloid protein precursor (APP) ranging in size from 677-770 aa (Goldgaber *et al.* 1987a, Goldgaber *et al.* 1987b, Kang *et al.* 1987, Robakis *et al.* 1987, Tanzi *et al.* 1987, Monning *et al.* 1992). Thus, A $\beta$  starts as a much larger protein that is subsequently proteolytically processed by multiple pathways. MAPT, on the other hand, had been discovered previously as a promoter of microtubule assembly (Weingarten *et al.* 1975). Although MAPT was found in several dementias and has been linked genetically to tauopathy-associated dementias, an early genetic association between APP and familial AD (FAD) brought APP to the center of AD research (Goate *et al.* 1991, Mullan *et al.* 1992). These genetic studies were complemented by biochemical findings that levels of longer 42 aa forms of A $\beta$  increase in FAD mutant cells. In addition, a major risk factor in AD is the  $\epsilon$ 4 allele of Apolipoprotein E (ApoE4). Several studies suggest that ApoE facilitates A $\beta$  deposition and that its  $\epsilon$ 4 isoform has a higher affinity for A $\beta$ . Based on studies showing that these longer forms of A $\beta$  readily aggregate into neurotoxic oligomers and fibrils in vitro, the fundamental hypothesis to describe the origin of AD has been that A $\beta$  initiates a toxic cascade that causes AD (Hardy and Selkoe, 2002).

This hypothesis has propelled the pharmaceutical industry to treat AD by reducing A $\beta$ . Because AD has helped investigators define a new phenomenon of protein aggregation and deposition that has been repeatedly observed in other neurodegenerative disorders such as Parkinson's disease (PD) and Huntington's disease (HD), it is hoped that the treatment paradigms developed for AD can be extended to other diseases.

Several agents were thus developed to reduce A $\beta$  aggregates in animal models, including one specific for A $\beta$ 42, but all of these lack efficacy in disease treatment (e.g. Alzhemed<sup>TM</sup>, Flurizan, <http://www.alzforum.org/drg/drc/default.asp>). However, it is important to note that most of these treatments failed to detect changes in A $\beta$  in the cerebrospinal fluid (CSF), leading scientists to speculate that the problem has been one of dosing (Green *et al.* 2009, Imbimbo 2009). Two agents that clearly reduce A $\beta$  in clinical trials are a vaccine against deposited amyloid - AN-1792, and a  $\gamma$ -secretase inhibitor (Portelius *et al.* 2010, Gilman *et al.* 2005). The former was removed from clinical trials when patients developed encephalitis due to the vaccine side effects although the approach is still being pursued using an alternative strategy of passive immunization (Klyubin *et al.* 2008, Bard *et al.* 2003). Also, in patients who did not acquire encephalitis, only showed marginal disease arrest was observed (Gilman *et al.* 2005). More recently, Eli Lilly has stopped trials using its  $\gamma$ -secretase inhibitor – semagacestat – as this drug performed less well than placebo (Extance 2010, Panza *et al.* 2010). Major arguments in the AD field are that drug failure is due to effects on an alternative and critical target, Notch, shifting the treatment focus to more selective inhibitors that preferentially inhibit APP processing while avoiding Notch. It is also important to note that function of APP and the role played by the secretases is not known making effects on the target unclear. Recent studies suggest that APP may play a role in innate immunity, a response affected by  $\gamma$ -secretase inhibition (Lanz *et al.* 2006, Jayadev *et al.* 2010).

When assessing the failure of these drugs in human clinical trials, one must consider several problems for achieving efficacy in AD drug development. First, we must extend research beyond amyloid-lowering agents, determining the best time for treatment along with alternative risk factors and target AD markers more proximal to the observed neurodegeneration.

First, the role of protein aggregation in degeneration and mechanisms of toxicity have not been fully established. Therefore, we will first discuss the evidence that supports the amyloid hypothesis and continue to argue in support of an alternative hypothesis: the failure of  $\gamma$ -secretase processing leads to AD pathogenesis. This theory is based on our findings that A $\beta$ 42 levels actually increase upon reducing  $\gamma$ -secretase (Refolo *et al.* 1999, Sambamurti *et al.* 2006, Marlow *et al.* 2003). The major prediction of this hypothesis is that inhibitors of  $\gamma$ -secretase will actually worsen rather than attenuate neurodegeneration in AD, because of the enzyme's important role in maintaining membrane homeostasis, failure of which, is the cause of AD-associated neurodegeneration. Such worsening of dementia was indeed observed in the trial (Extance 2010).

Other points to consider are that A $\beta$  dyshomeostasis is an early phenomenon and the dementia may be driven by other yet to be discovered lesions as well as dysfunction of other cell components such as APOE, MAPT, TDP43, synuclein, etc (Wollmer 2010, Gabelle *et al.* 2010). Thus, multiple proteins are misprocessed in AD, and this may lead to dementia by multiple pathways. Although these downstream events are critical to AD pathogenesis, we will be focusing on the A $\beta$ -related pathways to keep the discussion focused on drug development against this target.

A third problem is one of timing. For example, a known problem in genetic deficits in amino acid metabolism that leads to mental retardation is that compensation for the defect after the mental retardation sets in does not restore mental function (Orendac *et al.* 2003). Similarly, one may need to treat the problem of A $\beta$  accumulation before the cascade of events cause a failure of processing and function of other proteins that are ultimately catastrophic. Although it has been reported that AD pathology predates dementia by 10 years or longer, this remains controversial due to its high variability and lack of longitudinal studies that are now underway through ADNI (Caroli & Frisoni 2010). However, it is important to recognize that dementia advances quite rapidly once it begins, suggesting that the progression of dementia may be determined by factors other than A $\beta$  (Tarawneh & Holtzman 2009).

In short, given the recently reported failure of semagacestat and its withdrawal from clinical trials, it is critical to reevaluate the beneficial and detrimental effects of various A $\beta$ -reducing treatment strategies and discuss other potential targets for treatment.

## A $\beta$ and APP processing

APP is a large type-I, single-pass integral membrane glycoprotein with a large N-terminal ectodomain, a single transmembrane domain and a cytoplasmic domain of 47 aa (Hardy & Selkoe 2002, Sambamurti *et al.* 2006, Sambamurti *et al.* 2002b, Sambamurti *et al.* 2002a). Alternative splicing of three exons (7, 8, 15) within the ectodomain region of a single gene on chromosome 21 generates eight isoforms of APP (695–770 aa) that are differentially expressed in different cell types (Sambamurti *et al.* 2002b, Sambamurti *et al.* 2002a). The major neuronal form is APP695, which defines neuron-specific alternative splicing, lacks exon 7: a Kunitz protease inhibitor (KPI; 289-344) seen in APP751 and APP770 and exon 8: the OX2 (345-363) seen in APP770. Exon 15 (637-654) is also differentially spliced and is absent in lymphocyte APP (LAPP) that is absent in neurons (Sandbrink *et al.* 1994). Loss of this 18-aa domain generates a novel motif that gets decorated by chondroitin sulfate glycosaminoglycans (GAGs) (Robakis *et al.* 1993).

To generate A $\beta$ , APP is cleaved on the N-terminal side of the A $\beta$  sequence by  $\beta$ -secretase to secrete the ectodomain fragment sAPP $\beta$  and the remaining membrane-bound CTF $\beta$  is further processed by  $\gamma$ -secretase within the membrane to secrete A $\beta$  (Fig 1). However, this pathway accounts for the turnover of only a small fraction of APP and the majority (>90%)

of APP is processed by an alternate pathway,  $\alpha$ -secretase, between residues 16 and 17 of A $\beta$  to secreted derivative, sAPP $\alpha$ , that preclude A $\beta$  formation (Haass & Selkoe 1993, Sambamurti *et al.* 1992b, Anderson *et al.* 1991, Esch *et al.* 1990). This event is very rapid and occurs in the secretory pathway of the trans-Golgi network and on the cell surface (Sambamurti *et al.* 1992a, Sambamurti *et al.* 1992b).

The C-terminal fragments CTF $\beta$  and CTF $\alpha$  generated by  $\beta$ - and  $\alpha$ -secretase cleavages remain in the membrane and are processed by  $\gamma$ -secretase to generate secreted derivatives A $\beta$  and A $\alpha$  (A $\beta$ 17-40/42; aka P3) (Haass *et al.* 1993) and CTF $\gamma$  (aka CTF $\epsilon$  or AICD) (Pinnix *et al.* 2001) of 50 residues (Sastre *et al.* 2001, Gu *et al.* 2001). The A $\beta$  and A $\alpha$  fragments predominantly end at residue 40, while other forms, including A $\beta$ 42 are present at much lower levels (Seubert *et al.* 1992, Cai *et al.* 1993). The major importance of this finding has been that longer A $\beta$ 42 are increased as a general rule for most of the FAD mutations on APP (Suzuki *et al.* 1994).

The increase in A $\beta$ 42 was further supported and expanded to include FAD mutations linked to the critical  $\gamma$ -secretase subunit presenilin(s), PS1 and PS2 (Sherrington *et al.* 1995, Borchelt *et al.* 1996, Duff *et al.* 1996). These studies also confirmed a connection between APP and MAPT, as FAD mutations lead to AD earlier in life, with a richer neuropathological lesion load of typical plaques and tangles, even though the mutations only directly affect A $\beta$ 42 production before the development of pathology.

## Amyloid aggregation and toxicity

A large body of literature has focused on amyloid neurotoxicity, a phenomenon first discovered by Yankner and colleagues in 1990 (Yankner *et al.* 1990). It was proposed that aggregates of A $\beta$  induce toxic oxidative stress (Pappolla, *Am J Pathol* 1992). At that time, several well-known investigators questioned the data (Podlisny *et al.* 1992) and an entire issue of the journal *Neurobiology of aging* was dedicated to resolve the controversy (Yankner 1992). The conclusion of these studies was that amyloid is indeed toxic but that the toxicity varied wildly between preparations (May *et al.* 1992). Many studies showed that A $\beta$  readily forms oligomers and larger aggregates that are toxic to cells in culture (Walsh & Selkoe 2004, Chromy *et al.* 2003, Lambert *et al.* 1994, Glabe 2008, Pike *et al.* 1991).

The aggregation of synthetic A $\beta$  in vitro has been studied since the identification of the peptide in senile plaques but evolved into a larger discipline with the discovery that soluble oligomers rather than large insoluble aggregates of A $\beta$  are toxic (Klein *et al.* 2001). This theory explains the neuronal deficiency in APP transgenic mouse models that occurs long before apparent amyloid deposition is observed (Reed *et al.* 2009). Furthermore, the theory also accounts for the role of FAD mutations due to the greater tendency of the longer A $\beta$ 42 species to aggregate, compared to the shorter A $\beta$ 40. Finally, this theory can be supported by the generally accepted view that the ApoE4 facilitates aggregation of A $\beta$  and therefore increases the risk of AD (Fryer *et al.* 2005, Bales *et al.* 1999). This concept has gained substantive support from most investigators who suggested that A $\beta$  oligomers can affect multiple pathways in the brain such as insulin signaling, NMDA receptor activity, mitochondrial copy number and function and toxicity due to oxidative stress-mediated damage to neurons (Smith *et al.* 1998, Pappolla *et al.* 1998, Casadesus *et al.* 2007). The presence of toxic oligomers has been demonstrated in cell cultures and in the brain (Pappolla *et al.* 1998). The exact nature of the toxic oligomer is, however, still being debated and has ranged from dimer, trimer, tetramer, to dodecamer (Walsh *et al.* 2001, Reed *et al.* 2009, Freir *et al.* 2010). Nevertheless, the oligomers appear to induce a broad range of deficiencies, including effects on LTP formation and memory that can be specifically targeted by pharmacological manipulation. One group suggests that low concentrations of

A $\beta$  monomer and oligomer normally facilitates synaptic plasticity and memory but high concentrations lead to dysfunction and dementia (Fa *et al.* 2010, Origlia *et al.* 2009). A transgenic mouse model that expresses A $\beta$  oligomers lends additional support to this idea (Tomiya *et al.* 2010, Yankner 1992).

Mechanisms of A $\beta$ -induced toxicity remain rather unclear complicating the identification of downstream targets and their relevance to AD. Several targets have been discovered such as the insulin receptor, NMDA receptor, RAGE receptor, and ERAB (Klein 2002, Viola *et al.* 2008, Verdier *et al.* 2004, Origlia *et al.* 2008, He *et al.* 2000). Interestingly, the ERAB protein that binds A $\beta$  may enter mitochondria that provide additional possibilities for its disruption of cellular energy metabolism (Sambamurti & Lahiri 1998). It has been suggested that intracellular rather than extracellular A $\beta$  may actually be the cause of its toxicity (Tseng *et al.* 2004).

More recently, evidence suggests that a pyroglutamate form of A $\beta$  is the toxic species rather than simply the levels of the normal peptide (Wirhth *et al.* 2010). This has resulted in yet another target glutaminyl cyclase, the enzyme responsible for generating the pyroglutamate residue (Morawski *et al.* 2010).

## The amyloid hypothesis

The extremely early development of dementia and AD pathology in FAD also suggests that this pathology may be driven simply by A $\beta$  accumulation (Sambamurti *et al.* 2002b, Hardy & Selkoe 2002). The genetic link between A $\beta$ 42 and AD combined with the observed neurotoxicity *in vitro* form the basis for the amyloid hypothesis. Briefly, toxicity of A $\beta$  or its aggregates is considered to be the key cause of AD and this toxicity is believed to sequentially induce neuronal dysfunction, MAPT phosphorylation, NFT formation and neurodegeneration. The actual toxic agent has been a source of considerable controversy with theories shifting over time to include senile plaques to A $\beta$  aggregates of various types and the monomer. It has been argued that certain forms of A $\beta$  accumulate early in AD and induce the formation of NFTs by affecting the signaling cascade. Others suggest that A $\beta$  oligomers ranging from the dimer to the dodecamer are the toxic species that cause neurodegeneration in AD (Reed *et al.* 2009, Shankar *et al.* 2009). Also, some argue that microglia prevent oligomer toxicity but may induce toxicity when clearing plaques (Shah *et al.* 2010).

One of the early concerns of the amyloid hypothesis has been that senile plaques are frequently found in cognitively normal elderly individuals and the plaque count does not correlate with dementia (Terry 1998, Terry 1996). However, better correlation is observed when the plaque is limited to the neuritic kind and when soluble amyloid is measured (Parvathy *et al.* 2001). A significant advance that arose due to this controversy is an attempt to identify the toxic form of amyloid that may be responsible for AD pathogenesis.

Adhering to the amyloid hypothesis, one can readily find a number of potential targets for disease treatment (Table 1). Two key targets for inhibition in the synthesis pathway are  $\beta$ -secretase and  $\gamma$ -secretase. In addition, targets for stimulation are,  $\alpha$ -secretase, A $\beta$  degradation, and clearance pathways.

## Beta Secretase

The amount of A $\beta$  generated is apparently regulated by the extent of APP cleavage by  $\beta$ -secretase making it a target for inhibiting A $\beta$  production (Gandhi *et al.* 2004, Citron *et al.* 1992). The enzyme was identified by five groups as a novel single-pass, type-I integral membrane aspartyl protease and was variously named as BACE-1, memapsin-2 or Asp-2 by

the different groups (Yan *et al.* 1999, Vassar *et al.* 1999, Sinha *et al.* 1999, Hussain *et al.* 1999, Lin *et al.* 2000). Although memapsin-2 for membrane aspartyl protease is the most biochemically appropriate name, the most popular name in literature is BACE-1. The enzyme is not inhibited by the traditional aspartyl protease inhibitor, pepstatin, and apparently has a large catalytic pocket and has been a challenge to inhibit *in vivo* (Ghosh *et al.* 2008, Maillard *et al.* 2007). Moreover, BACE-1 is an evolutionarily conserved enzyme and the combined deletion of BACE-1 and its homologue BACE-2 increases neonatal lethality (Dominguez *et al.* 2005, Venugopal *et al.* 2008).

More recent studies have identified critical substrates for BACE-1 including neuregulin, LRP1, Klotho, sialyl transferase and voltage-regulated sodium channels (Li *et al.* 2010, Wong *et al.* 2005). In addition, a large number of substrates were identified by proteomic analysis recently (Hemming *et al.* 2009). These studies suggest that total inhibition of BACE1 *in vivo* may lead to unwanted reduction in the processing/turnover of a number of proteins that may lead to their toxic accumulation.

Although the nonspecific effects such as the impairment of other targets of BACE1 and  $\gamma$ -secretase may be considered to be critical for drug development, it is important to note that the partial inhibition of this type of processing may be well tolerated. For instance BACE1 heterozygous knockout mice, which express only 50% of the wild type activity are healthy (Roberds *et al.* 2001). Indeed, initial reports suggested that even 100% knockout in homozygous mice was tolerated: although, this finding was challenged in subsequent studies (Willem *et al.* 2006). Given that one can freely titrate the inhibitor dose, one may need to only partially inhibit APP processing to reduce A $\beta$  below a critical threshold. A benefit of inhibiting BACE1 is that, in addition to A $\beta$ , levels of CTF $\beta$  will also be inhibited. Several studies have suggested that CTF $\beta$  is also very toxic and will therefore be considered a second target (Neve *et al.* 1990).

A major caveat in this analysis is that BACE1 is an enzyme conserved from Dictyostelium, *C. elegans* and *Drosophila* to man, complicating the idea that it is an enzyme designed to constitutively generate large amounts of a toxic peptide. One would imagine that natural selection would eliminate this toxic property by mutating either the substrate or the enzyme in a variety of organisms. However, A $\beta$  and BACE1 target sequences on APP are remarkably conserved from reptiles to man (Table 2; excerpted from an NCBI Blast search of the NR database; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Therefore, A $\beta$  continuously generated and bound to other secreted proteins *in vivo* may either have a conserved function, or at least be a harmless secreted waste product. The cause of neurodegeneration may then be either the unnatural accumulated forms of the peptide, loss of some binding partner, or alternative metabolites whose homeostasis fails along with APP.

## Alpha Secretase

The absolute identity of  $\alpha$ -secretase remains unclear, although most evidence points to redundant processing by multiple members of the adamalysin family (ADAM-9, 10, 12, 17, 19). This pathway was referred to as the “good pathway” as it was technically anti-amyloidogenic and A $\beta$  is bisected into two pieces after residue 16. Nevertheless, it is important to note that inhibition of this activity does not increase A $\beta$  (Gandhi *et al.* 2004). However, inducible forms of  $\alpha$ -secretase were detected early during the characterization of APP processing pathways (Caporaso *et al.* 1992). These studies found that stimulation of inducible  $\alpha$ -secretase cleavage reduced the yield of A $\beta$ , suggesting that agents such as muscarinic receptor agonists or protein kinase C activators may be used to constitutively increase sAPP $\alpha$  at the expense of sAPP $\beta$  and A $\beta$  (Nitsch *et al.* 2000, Nitsch *et al.* 1992). These drugs are currently being tested in clinical trials ([www.Alz.org](http://www.Alz.org)). Still, as part of a

powerful signaling cascade that may affect various critical cellular pathways, this approach may be challenging to consistently implement. Multiple membrane proteins are processed by  $\alpha$ -secretase like pathways, including most of the targets described listed in the  $\beta$ -secretase section. The pathway includes key developmental genes such as Notch and appears to be exquisitely regulated. While stimulating this process, it may be critical not to disturb the carefully regulated network of  $\alpha$ -secretase substrates. Finally, although A $\beta$  is traditionally treated as the toxic protein, truncated A $\beta$  (17-40/42) generated after  $\alpha$ - and  $\gamma$ -secretase cleavages are also reported to be toxic by forming calcium channels (Jang *et al.* 2010).

## $\gamma$ -secretase

Intramembrane proteolysis of several type-I integral membrane glycoproteins is mediated by  $\gamma$ -secretase, a multi-subunit protease complex containing several integral membrane proteins. Four essential subunits of this enzyme have been identified: Presenilin (PS1/PS2); anterior pharynx (APH-1); presenilin enhancer (PEN-2); nicastrin (NCT). Increasing the concentration of the four subunits in animal cells increases  $\gamma$ -secretase activity but increasing PS1 or PS2 alone does not (Marlow *et al.* 2003, Edbauer *et al.* 2003). Reconstitution of the four subunits in yeast, which normally lacks  $\gamma$ -secretase, generates the active enzyme, suggesting that these subunits are sufficient for activity, although other regulatory subunits may be present (Edbauer *et al.* 2003). The most well known substrate of  $\gamma$ -secretase is APP, which generates the secreted A $\beta$ 40 and 42 peptides from CTF $\beta$  as described earlier. The literature suggests that this essential enzyme gives rise to two toxic peptides, A $\beta$  and CTF $\gamma$ /AICD (Ghosal *et al.* 2009, Pimplikar 2009). An important recent alternative theory is that accumulation of CTF $\gamma$ /AICD causes neuronal dysfunction and MAPT hyperphosphorylation (Ghosal *et al.* 2009, Pimplikar 2009). However, it is important to note that the APP intracellular domain fragment expressed is actually 59 residues long rather than the normally found 50-residue fragment.

## Presenilin: Gain or loss of function

The major catalytic subunit of  $\gamma$ -secretase is PS1/PS2, which bears multiple mutations that cause FAD (Sambamurti *et al.* 2006, Sambamurti *et al.* 2002a). For a number of years, the argument has been that these mutations cause a novel gain of function to increase the ratio of A $\beta$ 42/A $\beta$ 40. However, a number of groups questioned the gain of function theory based on the hypofunction of mutant PS1 in *C. elegans* (Levitan *et al.* 1996). Some suggested that PS1 has alternative activities that may be compromised in AD such as a critical role in endocytosis (Tamboli *et al.* 2008, Ikeuchi *et al.* 2003).

Our studies using antisense RNA to inhibit PS1 expression showed that when PS1 levels got reduced to a level before detection, A $\beta$ 42 actually increases without significantly changing A $\beta$ 40 (Refolo *et al.* 1999). In contrast, when  $\gamma$ -secretase activity was increased by coexpressing all four subunits of the enzyme, A $\beta$ 42/A $\beta$ 40 ratios were reduced, an effect that is opposite of that seen by transfection of FAD mutation PS1 (Marlow *et al.* 2003).

All these studies indicate that FAD mutations lead to loss of function. Moreover, the loss of function appears to affect  $\gamma$ -secretase processing rather than an alternative function of PS1 or PS2.

## Mechanism of $\gamma$ -secretase cleavage

Several groups showed that  $\gamma$ -secretase cleavage proceeds sequentially (Fig 2) by first cleaving at the membrane cytoplasmic junction of APP to generate A $\beta$  of 49 residues (Xu 2009, Takami *et al.* 2009). This fragment is rapidly cleaved to A $\beta$ 46 by the same enzyme (Zhao *et al.* 2004). Interestingly, these cleavages are inhibited by transition state inhibitors

such as LY-685,458 but N-[N-(3,5-difluorophenacetyl)-L-alanyl]-L-(S)-phenylglycine methyl ester; DAPT, allow the first two cleavages to proceed resulting in the accumulation of A $\beta$ 46, which is subsequently sequentially processed to A $\beta$ 40 and A $\beta$ 42 (Zhao *et al.* 2010).

This finding fits with our hypothesis that CTF $\alpha/\beta$  is cleaved close to the membrane cytoplasmic junction first, where it can access the water from the cytoplasm for hydrolysis (Sambamurti *et al.* 2006). The remaining transmembrane domain floats freely within the membrane to be cleaved at the membrane/lumen junction. Unlike the original cut-expose-cut model (Murphy *et al.* 2002), the protein will not require extraction from the membrane for cleavage.

An early finding is that inhibition of  $\gamma$ -secretase results in substrate accumulation in the membrane and yields more cleavage products upon incubation of these membranes (McLendon *et al.* 2000). In addition, our unpublished results (Venugopal and Sambamurti) show that CTF $\alpha/\beta$  turnover is extremely slow upon inhibition of  $\gamma$ -secretase, suggesting that the cell lacks efficient alternative pathways for its disposal. Further, initial long exposure followed by removal of  $\gamma$ -secretase from the medium allows the accumulated CTF $\alpha/\beta$  to turnover suggesting that the pathway may generate A $\beta$  from accumulated substrate (Sharma, Barnwell and Sambamurti, in preparation). This may explain the large increase in A $\beta$  reported in later time points after  $\gamma$ -secretase inhibition in human clinical trials (Siemers *et al.* 2007). Indeed this type of generation of A $\beta$  in bursts may facilitate seeding of the A $\beta$  oligomers and fibrils providing yet another A $\beta$ -related toxic mechanism by partial or intermittent  $\gamma$ -secretase inhibition.

Several studies have suggested that loss of  $\gamma$ -secretase activity increases the frequency of cancer through unknown mechanisms (Das *et al.* 2004, Roperch *et al.* 1998). Recent studies suggest that mutations that dramatically lower  $\gamma$ -secretase activity may be linked to an unusually aggressive form of Acne inversa (Wang *et al.* 2010).

Thus, even if AD is triggered by accumulation of toxic A $\beta$  oligomers,  $\gamma$ -secretase inhibition may be a poor option to pursue for disease treatment given that it may actually facilitate the formation of A $\beta$  aggregates and given its large number of essential substrates.

## Mouse models

Despite the failure of transgenic mice to truly model the pathology of AD with progressive synaptic loss followed by neurodegeneration of cholinergic and hippocampal neurons, the partial pathology showing amyloid deposition as plaques and behavior deficits has made it a useful model for at least partly understanding the role of A $\beta$  and PS1/PS2 mutations in neuronal dysfunction (Games *et al.* 1995, Cheng *et al.* 2007).

## Limitations of the current animal models to study AD

A major argument against the amyloid hypothesis has been that senile plaques develop in transgenic mice without concomitant neurofibrillary tangle formation or neurodegeneration. However, one needs to appreciate that the mice express mutant forms of APP and PS1 that are linked to FAD, and therefore failure to find appropriate neurodegeneration in these mice simply indicates that the model is unable to develop AD pathology and cannot prove or disprove any hypothesis. Part of the problem with animal models is that evolution retains the physiologically critical elements across various phyla, but pathology does not have to be conserved unless mandated by limitations of the shared design.

In addition to mutations that modulate APP processing to influence A $\beta$ 42 yield, a simple duplication of the APP gene resulting in a 1.5-fold increase in its copy number has been a

reported to be a cause of FAD (Rovelet-Lecrux *et al.* 2007). This also explains the nearly inevitable AD pathology in older Down's syndrome patients. It is important to note a caveat at this stage: although APP transgenic mice expressing high levels of amyloid fail to show the characteristic loss of cholinergic neurons, trisomic mice that model Down's syndrome do show this loss (Lockrow *et al.* 2009, Salehi *et al.* 2006). Furthermore, the loss of cholinergic neurons can be prevented if one of the copies of APP is knocked out, suggesting that factors other than APP in the mouse chromosome 16 (syntenic with 21 in humans) may facilitate the APP-mediated neurodegeneration. Interestingly, all these changes appear to be mediated by a simple 1.5-fold increase in APP and its CTFs without any obvious deposition of amyloid. This mouse model also shows an APP dose-dependent increase in degeneration of the locus coeruleus beta-adrenergic neurons (Heneka *et al.* 2002), which has recently been recognized an important pathway that is lost early in AD. Interestingly, cholinergic and adrenergic neurons appear to play a parallel role in memory and one may compensate for the other in young animal models (Wenk *et al.* 1987). This neuronal pathway may therefore be targeted like the cholinergic pathway for treatment of AD.

Certain caveats associated with the aforementioned animal models exist and should be emphasized. First, the mechanism of accelerated aging in these models are either unknown or associated with specific targeted genetic manipulations that may not fully recapitulate the processes that occur during physiological aging. APP regulation differs between human and the widely used animal models. For example, the APP5'-UTR, including the important CAGA box, differs significantly between plaque-forming and non plaque-forming species, among 26 different species studied (Maloney *et al.* 2004). In addition, polymorphisms of the APP and APOE promoters implicated in AD etiology do not have corresponding homologous sequences in the mouse or rat gene promoters (Lahiri *et al.* 2005). In other words, sufficient evidence exists to indicate the folly of merely presuming that AD associated gene regulation at any level would be identical

## **An alternative model suggesting that $\gamma$ -secretase inhibition causes AD**

We have previously proposed that failure of  $\gamma$ -secretase is the cause of AD in families carrying FAD mutations (Sambamurti et al 2006). We will discuss some of the aspects of the hypothesis below.

### **Partial $\gamma$ -secretase inhibition by FAD mutations**

An important part of  $\gamma$ -secretase processing of APP is that several inhibitors act quite anomalously showing increases in A $\beta$ 42 at low doses. Indeed, it was initially proposed that different enzyme generate A $\beta$ 42 and A $\beta$ 40 (Figueiredo-Pereira *et al.* 1999). However, this was ruled out as subsequent studies found that several inhibitors actually increase A $\beta$ 42 at low doses (Durkin *et al.* 1999). This phenomenon was confirmed when we determined that PS1 antisense RNA reduces PS1 and increases A $\beta$ 42 while slightly and non-significantly reducing A $\beta$ 40 (Refolo *et al.* 1999), and others found that knocking PS1 out results in a complete loss of  $\gamma$ -secretase cleavage and A $\beta$ 40 as well as A $\beta$ 42 (De Strooper *et al.* 1998). Finally, we found that a combination of limiting subunits – Aph1, Pen2 and nicastrin – resulted in increased  $\gamma$ -secretase processing but also reduced AB42/A $\beta$ 40 ratios (Marlow *et al.* 2003).

As described earlier, recent studies have found that  $\gamma$ -secretase actually includes two independent activities that are inhibited by different compounds. The same enzyme however sequentially cleaves CTF $\beta$  in increments of three until it reaches A $\beta$ 40/A $\beta$ 42 as repeatedly demonstrated by multiple groups (Takami *et al.* 2009, Xu 2009). Sequential cleavage of A $\beta$  by  $\gamma$ -secretase adds further support to the partial  $\gamma$ -secretase inhibition hypothesis and supports a second new hypothesis regarding the mechanism of intramembrane proteolysis

(Fig 2). Briefly, it takes four cleavage steps from 49-46-43-40 to A $\beta$ 40 but only three steps to A $\beta$ 42 (48/49-45/46-42). These cleavages yield products that are predominantly trapped within the membrane/ $\gamma$ -secretase cleft and are in constant equilibrium with the lumen/medium. When the peptide gets released into the medium it is diluted and therefore no longer part of the cleavage reaction. Therefore, slow or impaired second site cleavage will result in an increase in A $\beta$ 42/43, which will be released before it gets cleaved further to A $\beta$ 40.

One study of a large number of FAD mutants showed that some mutations reduce CTF $\gamma$  but others do not have such dramatic effects (Walker *et al.* 2005). These findings can be readily explained by the presence of two different catalytic activities in  $\gamma$ -secretase that may be independently affected by the FAD mutations, as discussed earlier.

Thus the idea of using  $\gamma$ -secretase as a treatment target may be ideal, if one proposes that it is the enzyme that generates two toxic peptides. However, if we were to work from the perspective that it is an essential enzyme with numerous substrates ranging from Notch and Cadherin to Klotho and neuregulin, we will need to be cautious about using inhibitors to treat the disease (Sambamurti *et al.* 2006). Even if one finds an inhibitor that does not cleave Notch, it may still inhibit the processing of other essential substrates. The failure of treatment may readily find scapegoats in targets other than Notch and the importance of the enzyme in preventing AD may be lost.

We have proposed that since large amounts of APP are synthesized and turned over by this pathway continuously, and the protein is preferentially trafficked into axons and dendrites, its metabolites may simply accumulate in nerve endings and prevent the recovery and reuse of synaptic membranes. This should in turn result in traffic jam of membrane vesicles with reduced recycling capabilities. The failure to recycle membranes may then lead to the synaptic dysfunction observed in AD even before loss of cells. Axonal transport failure may then lead to MAPT accumulation in the cell body where it is mislocalized and deposited as NFTs (Sambamurti *et al.* 2006).

### Other hypotheses pertaining to $\gamma$ -secretase dysfunction

The basis of our theory that  $\gamma$ -secretase impairment causes AD is that direct antisense RNA-mediated inhibition of PS1 synthesis led to an increase in the A $\beta$ 42/40 ratio (Refolo *et al.* 1999) and drop in the A $\beta$ 42/A $\beta$ 40 ratio upon reconstitution of  $\gamma$ -secretase activity by coexpression of all four subunits (Marlow *et al.* 2003). The notion was supported by findings that FAD mutant PS1 was unable to complement sel12 mutations in *C. elegans* (Levitan *et al.* 1996).

Several groups suggested that PS1 had functions independent of  $\gamma$ -secretase such as intracellular adhesion and capacitative calcium entry that may explain its hypofunction while simultaneously explaining a gain of function with respect to  $\gamma$ -secretase activity (Saura *et al.* 2004, Yoo *et al.* 2000, Singh *et al.* 2001). Thus a theory was promulgated that presenilin loss of function causes neurodegeneration, but this dysfunction may be due to multiple functions of the protein (Shen & Kelleher 2007). The discussion was supported by findings that loss of PS1 or nicastrin, two  $\gamma$ -secretase subunits, leads to neuronal loss and behavior deficits in mice (Saura *et al.* 2005, Tabuchi *et al.* 2009, Lambert *et al.* 2001). This proposed hypothesis includes discussion suggesting that  $\gamma$ -secretase hypofunction may also be a basis for AD but that additional loss of alternative function may also play a role. The key points in this hypothesis are also highlighted by other studies showing that FAD mutations affect cadherin processing by  $\gamma$ -secretase and affect the yield of the critical memory-related factor – CREB (Marambaud *et al.* 2003).

Reliance on behavior or neurodegeneration as endpoints of genetic manipulation in AD mouse models is problematic, as they may be broadly affected by a number of factors such as loss of one or more essential functions of presenilin, altered blood supply, neurotransmitter imbalance, inflammation, etc. Thus, a lot of nonspecific pathologies may lead to behavior deficits, which may not represent a result of logical development of AD-specific pathology. Behavior improvement in mice has been repeatedly demonstrated with  $\gamma$ -secretase inhibitors and other A $\beta$ 42-centric treatments, without their successful translation to humans (Spilman *et al.* 2008, Comery *et al.* 2005, Lambert *et al.* 2001). Nevertheless, these findings indicate that the behavioral impairment in mice that are engineered to express high levels of A $\beta$ 42 is due primarily to A $\beta$ -dependent pathology.

Recent studies have identified mutations and variants in  $\gamma$ -secretase subunits that may provide some insight into the role of this enzyme in AD (Wang *et al.* 2010, Coolen *et al.* 2006, Coolen *et al.* 2005). The Coolen *et al.* study is noteworthy and suggests that  $\gamma$ -secretase loss leads to specific neuronal deficits as Aph-1b loss leads to specific apomorphine (dopamine agonist) hypersensitivity.

The role of reduction in  $\gamma$ -secretase activity in AD has been questioned as the  $\gamma$ -secretase hypofunction mutations (frameshifts, nonsense) observed on PS-1, PEN-2 and NCT were not associated with an increase in incidence of AD (although further studies are needed to exclude disease association) and acne inversa was not observed in AD (Wang *et al.* 2010, Kelleher & Shen 2010).

How can we reconcile these findings with our model of membrane protein homeostasis and PS1 loss of function? It is critical to note that few of these acne inversa patients are very old and hence, more studies are needed to confirm any association and the study did not report any activity measurements (Wang *et al.* 2010). Loss of function mutations in the heterozygous state do not normally induce a large loss of activity in multi-subunit enzymes of this type as the defective proteins are not incorporated into the complex, but only affect the rate of assembly. In contrast, missense mutant proteins do incorporate into the complexes and generate defective enzymes. Extreme loss of  $\gamma$ -secretase is incompatible with life leading to embryonic lethality and is therefore unlikely to be encountered in adults. Moreover, the reported mutations are likely to produce significant levels of truncated proteins that may inappropriately interact with substrates or other proteins to produce dysfunctional protein complexes. Acne inversa or hidradenitis suppurativa occurs in skin creases due to blockage of sweat glands and inflammation, likely caused by accumulation such defective protein complexes. Alternatively, mutations may increase susceptibility to acne inversa by developmental failure of sweat glands and sebaceous glands within hair follicles. Further, detailed analysis is definitely needed to understand the biochemical basis of the observed acne inversa phenotype and its relationship to AD.

Secondly, because multiple forms of  $\gamma$ -secretase manifest due to the existence of PS1, PS2 and multiple variants of its Aph-1 subunits, and have different substrates in different tissues, the behavior of these mutations can vary considerably with organ systems, a factor that also needs additional investigation. Heterozygous knockout PS1, PS2 and other  $\gamma$ -secretase subunit mice are indeed viable. Recent studies indicate that even a complete loss of NCT allows generation of active  $\gamma$ -secretases (Zhao *et al.* 2010).

Third, our hypothesis is not dependent on developmental losses that arise due to loss of  $\gamma$ -secretase function, but a malfunction that occurs in older people who still need an active enzyme to maintain membrane function and homeostasis in the brain. APP, in our opinion, is a key player because it is highly expressed in the synapse and has a tendency to strongly interact to form membrane-associated complexes with cytoplasmic and extracellular factors

that cannot be easily cleared. We propose that along with APP, other proteins such as lipoprotein receptors also fail, overwhelming the alternative maintenance mechanisms that preserve synaptic integrity. Intermittent failure of  $\gamma$ -secretase due to physiological or environmental factors that overload the enzyme could actually foster amyloid deposition, as the substrate, CTF $\beta$ , will accumulate in the absence of its rapid turnover, and yield bursts of A $\beta$  that are concentrated in membranes, to promote seeding and aggregation. In particular, the long axons need to be supplied by an active microtubule system with a local management of membranes at the presynaptic terminals that will be stressed by such a failure resulting in a large biochemical requirement for synaptic membrane replenishment. This in turn will lead to disruption of the microtubule-MAPT structure in axons. The difference in AD susceptibility of different species may simply be a reflection of the large expansion of the human brain leading to reduced adaptability to such extreme biochemical requirements. Several factors that may be explained by this hypothesis include MAPT failure, the association with the ApoE  $\epsilon$ 4 allele and the loss of brain cholesterol associated with AD (Sambamurti et al. 2006).

**Predictions of the  $\gamma$ -secretase inhibition model for AD**—Some of the original predictions of this model were as follows:

1. Membranes from dystrophic neurons should show an accumulation of APP CTFs
2. We should see accumulation of other  $\gamma$ -secretase substrates in the degenerating brain.
3. FAD mutations should disrupt or inhibit other functions of  $\gamma$ -secretase substrates such as cholesterol homeostasis.
4. Chronic sublethal inhibition of  $\gamma$ -secretase should cause neurodegeneration in appropriate models systems to augment AD dementia.

The failure of the recent Ely Lily clinical trial provides validation for this hypothesis by the conducting the experiment and proving proof for prediction number 4. Although evidence does exist for the other prediction, we need more studies to provide direct support.

Several factors such as high cholesterol diets are believed to cause AD and a number of studies support the idea that cholesterol increases amyloid deposition (Pappolla *et al.* 2002, Refolo *et al.* 2001, Refolo *et al.* 2000, Sambamurti et al. 2004). We have found that isoprenoids that are synthesized in the cholesterol biosynthesis pathway are necessary for  $\gamma$ -secretase assembly (Zhou *et al.* 2008). High cholesterol is known to inhibit the synthesis of mevalonic acid, which is the precursor of isoprenoids and may therefore impair  $\gamma$ -secretase assembly. These predictions need to be tested in animal models and in human clinical samples. The major brain cholesterol transporter is ApoE and may play a role in this regulation. Moreover, the LDL and LRP receptors that are known to transport ApoE are also  $\gamma$ -secretase substrates that may create imbalances in the enzyme-substrate ratios.

## Conclusion

AD is a complex neurodegenerative disease that takes a number of years to develop. Current animal models are limited, as they do not truly follow the complex neuropathology and neurodegeneration observed in subjects with AD. Indeed, a number of people with AD neuropathology are cognitively normal raising the possibility that the lesions do not necessarily lead to disease in everyone. The transgenic mice may be better models of this type of pathological aging. The amyloid hypothesis is complicated by the identification of multiple toxic forms of A $\beta$  and multiple toxic domains on APP (Fig 3). Furthermore, multiple toxic proteins found in other neurodegenerative diseases also accumulate in AD

and may contribute to the neurodegeneration in the disease (Fig 4). The major support for the amyloid hypothesis comes from the increased production of A $\beta$ 42 by FAD mutations and toxicity of oligomeric aggregates. Our analysis in this article indicates why this may not be a gain of pathological function, but may actually represent inhibition of  $\gamma$ -secretase activity and therefore a loss of function series of mutations. While we are not contradicting the amyloid hypothesis, we propose a modified version of the amyloid hypothesis that takes a broader view of misprocessed proteins and suggests that neurotoxicity may involve a host of  $\gamma$ -secretase substrates beyond amyloid. We propose that loss of  $\gamma$ -secretase activity leads to accumulation of several membrane protein fragments to different degrees, depending on the extent of  $\alpha$ - and  $\beta$ -secretase like processing of these substrates. Individually this increase may be small and undetectable except for highly expressed and processed proteins like APP, but the combined accumulation of membrane-bound stubs will likely make the membrane unavailable for recovery and recycle, and ultimately lead to synaptic dysfunction. In short, the pathological pathway in AD is complex, and like cancer, we may see the unraveling of multiple mechanisms of degeneration with benefits that will extend to multiple diseases in the process. However, the cautionary note may be that we may need to expand the basic research in the area enormously to tackle the complex problem and limit translational studies to those that can be well validated.

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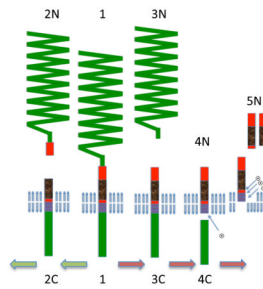
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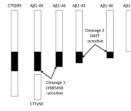
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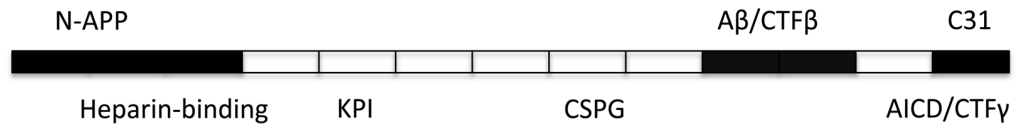
**Figure 1. Pathways of APP proteolysis**

The full-length APP is a type-I integral membrane protein. It is cleaved in the exocyttoplasmic domain at the start of A $\beta$  by  $\beta$ -secretase and between residues 16-17 of A $\beta$  by  $\alpha$  secretase to generate secreted derivatives sAPP $\beta$  and sAPP $\alpha$  and C-terminal fragments CTF $\beta$  and CTF $\alpha$ .  $\gamma$ -Secretase cleaves CTF $\beta$  at the membrane cytoplasm boundary ( $\epsilon$  site) to CTF $\gamma$  and A $\beta$ 49, and subsequently to A $\beta$ 40 and A $\beta$ 42. Most secreted A $\beta$  ends at residue 40 but a small percentage is longer terminating at residues 42 and 43. CTF $\alpha$  is similarly processed by  $\gamma$ -secretase (not shown). The predicted CTF $\gamma$  is not seen probably due to its rapid turnover in the cell.



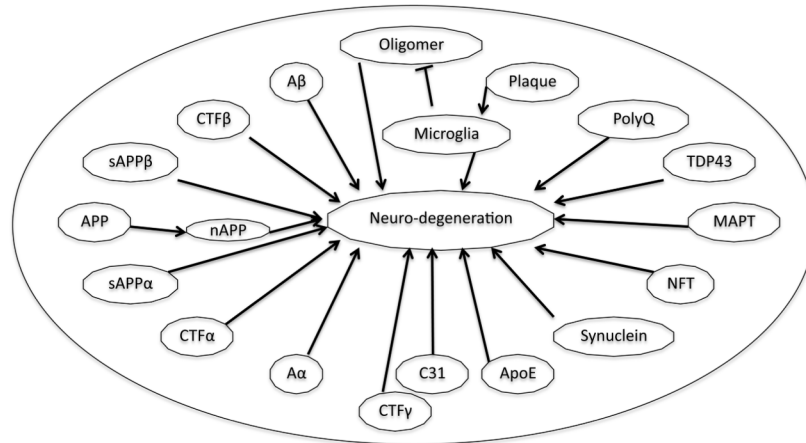
**Figure 2. Model for  $\gamma$ -secretase processing of APP**

The final step of intramembrane processing is mediated by  $\gamma$ -secretase, which is a multisubunit integral membrane protein. Cleavage of membrane protein is enigmatic due to the lack of water within the membrane for hydrolysis. The proposed model takes advantage of the knowledge that  $\gamma$ -secretase is a multicatalytic enzyme that cleaves CTF $\alpha/\beta$  multiple times within the membrane and these cleavages are differentially sensitive to two different inhibitors: LY-685,458 and DAPT. The hypothesis suggests that after cleaving at the membrane cytoplasmic boundary, the transmembrane domain is still bound to the first LY-685,458 – sensitive active site of the enzyme and is subsequently free floating within the membrane. At this stage, it is susceptible to cleavage at the membrane - medium interface where it is cleaved by the second DAPT-sensitive active site. Thus, LY-685,458 blocks all cleavages whereas DAPT only blocks the cleavage from A $\beta$ 46 to A $\beta$ 43 and beyond. Each of these cuts are slightly ragged, but normally follow a pattern of cleaving three residues from the first cleavage.



**Figure 3. Multiple toxic domains and metabolites of APP have been defined**

Multiple cleavage sites have been defined on APP and these appear to generate a number of toxic proteins such as n-APP, A $\beta$  and C31 shown above the APP molecule in the schematic diagram (not to scale). Even the major cleavage products such as CTF $\gamma$ /AICD have been defined as toxic in animal models. These potentially toxic fragments demonstrate that the toxicity of APP may arise due to improper turnover and cleavage rather than due to the normal turnover pathways and argue against disrupting normal turnover mechanisms. APP is an important multifunctional protein with the key known functional domains shown below the molecule.



Failure of membrane protein processing and turnover

**Figure 4. Faulty protein turnover can lead to toxic accumulation of a number of proteins**

A number of proteins are known to accumulate in senile plaques and neurofibrillary tangles in subpopulations of patients with AD. Several of these proteins are known to accumulate in other degenerative diseases and have mutations linked to the disease. Some of the accumulated proteins become toxic after microglial processing whereas others are degraded to inactive products by microglia as shown for plaque amyloid. None of these proteins are neurotoxic in the young or even most of the health aging populations. Thus, rather than focusing on toxic products one may need to find ways to preserve the proteolytic clearance pathways that maintain secreted, membrane and cytoplasmic protein homeostasis.

**Table 1**

Major drug targets based on the amyloid hypothesis

Target	+/-	Examples	Side effects of specific drug
APP synthesis	-	Posiphen; Antisense; siRNA	Limited! APP is redundant
$\beta$ -secretase	-	CTS21166	BACE1 has multiple substrates
$\gamma$ -secretase*	-	semagacestat	Multiple targets, Toxic substrate accumulation
$\alpha$ -secretase	+	Benzolactam	Complex and not yet defined. Target dependent
A $\beta$ degradation	+	TPA, UPA	Limited! May be very specific.
A $\beta$ clearance	+	ApoE, LRP	Complex and not yet defined. Target dependent
A $\beta$ aggregation	-	PBT-2	Limited! May be very specific.
Vaccination	+	Vaccination; Passive immunization	Inflammation
A $\beta$ toxicity	-	Anti-Tau; -oxidants; -inflammatory	Complex and not yet defined. Target dependent

+ = Stimulate; - = Inhibit

\* Alternate hypothesis: Inhibitor may cause AD!

**Table 2**Sequence surrounding the  $\beta$ - and  $\alpha$ -secretase - cleavage sites are conserved

-14 to -1	$\beta$ -site 1-16	$\alpha$ -site 17-28	29-40	
LTNIKTEEISEVKM	DAEFRHDSGYEVHHQK	LVFFAEDVGSNK	GAIIGLMVGGVV	Homo <sup>1</sup>
LTNVKTEEVSEVKM	DAEFRHDSGYEVHHQK	LVFFAEDVGSNK	GAIIGLMVGGVV	Gallus <sup>2</sup>
LTNVKTEEISEVKM	DAEFRHDSGYEVHHQK	LVFFAEDVGSNK	GAIIGLMVGGVV	Chelydra <sup>3</sup>
LTNIKTEEISEVKM	DSEYRHDATAYEVHHQK	LVFFAEEVGSNK	GAIIGLMVGGVV	Xenopus <sup>4</sup>
LTGIKTEEIAELKM	ETEFQQDSGYEVHHQK	LVFFPKDVGSNK	GAIIGLMVGGVV	Narke <sup>5</sup>
VTGKSMEAVPELRM	ETEDRQSTEYEVHHQK	LVFFAEDVGSNK	GAIIGLMVGGVV	Tetraodon <sup>6</sup>

<sup>1</sup> Human,<sup>2</sup> Chicken,<sup>3</sup> Turtle,<sup>4</sup> Frog,<sup>5</sup> Electric ray,<sup>6</sup> Pufferfish