Transgenic Mice are not Valid for Late Onset Alzheimer’s Disease
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Abstract

Alzheimer’s disease is a major cause of morbidity and mortality in the elderly. It is characterized by a cognitive decline and the presence in the cerebral cortex of extracellular deposits of ß-amyloid (Aβ) in plaque and intracellular deposits of an hyperphosphorylated microskeleton protein, tau, in the form of tangles. It is commonly thought by a majority of workers in the field that the cognitive decline is due to either the neurotoxicity of the Aβ or the tangles. The early onset familial forms of the condition have a similar pathology and clinical course but are due to mutations in three proteins: amyloid precursor protein and presenilins 1 and 2. None of these mutations are found in the elderly. Yet in spite of their absence most workers believe that the cognitive decline seen in the early and late onset forms have the same underlying biochemical basis. As a result, they have developed mouse models transfected with mutant forms of the proteins associated with the early onset disease. These mice have been used to screen for potential therapeutic agents. Yet all 244 drugs that were found to be effective in these mice failed to show any benefit in clinical trials in elderly patients, including antibodies to the Aβ and inhibitors of the ß- and γ-secreases which are thought to be critical for plaque formation. These failures raise the question of whether the mouse models are valid test systems for drug discovery for treatment of the late onset disease. In this review I have outlined the biochemistry of Aβ production and studies from my laboratory that suggest that the cognitive decline is due to a decrease in the posttranslational processing in the endoplasmic reticulum of the synaptic membrane proteins that are necessary for a functioning memory. These findings suggest alternative models for the development of test systems for drug discovery to treat the late onset disease.

Keywords: Familial Alzheimer’s Disease; Late Onset Alzheimer’s Disease; Drug Discovery; Clinical Trials; Protein Folding; Endoplasmic Reticulum; N-Glycosylation, CSF.

As is widely recognized with the aging of the world’s population, late onset Alzheimer’s disease has become a major cause of morbidity and mortality in the elderly. In fact, in the United States it is now the third leading cause of mortality after cardiovascular disease and cancer. It also represents one of the leading financial burdens on our health care system. This burden, along with the stress which this disease places on both the patients and their families, has led to a consensus that there is a great need for research and development in the search for effective therapies. Recognizing the market potential of such treatments, the pharmaceutical industry has poured massive sums into drug development to fill this growing need. Yet, in spite of these vast sums, they have yet to develop any robust therapies to treat this condition. The magnitude of this failure is illustrated by the report by Cummings et al. (2011). These authors searched http://www.clinicaltrials.gov/ for all phase I-III trials in late onset Alzheimer’s disease patients. None of the 244 agents identified showed the progression of the disease in patients with mild to moderate Alzheimer’s disease. This consistent failure raises the question of whether the animal models currently used for drug screening are actually appropriate for seeking therapies for the late onset disease. He has recently updated his review with similar findings [1].

Currently the gold standards for drug development in Alzheimer’s disease are mouse models transfected with genes associated with the familial forms of the disease. The construction of these models is based on the proposition that the cognitive decline is due to the same biochemical mechanism in the early and late onset diseases. In spite of the failure of this approach to lead to the development of therapies that have a robust effect on the cognitive decline, both the academic and industrial communities have shown little interest in taking new approaches to develop screening systems for drug discovery. It is not surprising that the academic community is taking a conservative approach since their funding depends upon a variety of peer review processes. Reviewers tend to be very conservative and deny funding to programs that stray too far from the currently accepted paradigm. On the other hand, the pharmaceutical industry faces the opposite problem. Their funding depends entirely upon their ability to patent and produce marketable products. At the present time rather than directly funding innovated approaches, they have generally used their substantial funds to buy small biotech companies that have potentially marketable products. This approach has worked well in those areas where the cell biology of diseases, such as autoimmune, cancer or cardiovascular diseases, are well understood. But in a field like the treatment of late onset Alzheimer’s disease, it has yet to yield any major breakthroughs. Part of the problem is that most of the startups are extensions of well-known academic laboratories which have been well funded through research grants. As a result, their approach is based on the current paradigm, which in this case has yet to produce any agents which halt the progression of the disease in patients with mild to moderate cognitive loss. This narrow focus is a result of the historical development of our understanding of Alzheimer’s disease.

Although presenile dementia has been recognized since ancient times, it was not until 1906 when Alois Alzheimer, a German psychiatrist, described a patient, Auguste Deter, who was a 55-year-old woman who had had been institutionalized for several years as a result of disruptive behavior and cognitive decline. At autopsy Alzheimer employed staining techniques which he had developed with Franz...
Nissl. He described two neuropathological lesions characteristic of what was then called presenile dementia: extracellular deposits and intracellular tangles. In 1910 Emil Kraepelin, a German psychiatrist who worked with Dr. Alzheimer, first named this condition “Alzheimer’s Disease” in the eighth edition of his book *Psychiatrie*.

Concurrent with Alzheimer’s work Oskar Fisher, an investigator at the German University of Prague correlated the clinical course in a large number of patients with a variety of neurological diseases with the clinical findings and the observed neuropathology at autopsy. He concluded that the dementia of late onset disease was a result of plaque deposition [2]. Interestingly Alzheimer believed that these deposits were merely a biomarker for this condition. Their difference of opinion on the role of plaque in the development of dementia is a question that is still relevant today.

Robert Katzman [3], a well-known neurologist from the Einstein School of Medicine in the Bronx, NY, published an editorial in the *Archives of Neurology* in which he contended that based on the neuropathology and the clinical course, both presenile and senile dementia were the same disease described by Alzheimer. He further argued that since the late 1940’s this was a widely held opinion among neurologists.

Beginning in the 1980’s the core protein of the plaque, β-amyloid (Aβ) was purified and sequenced from the brains of patients with both late onset Alzheimer’s disease and Down’s syndrome [4,5]. Employing this sequence data both Tanzi et al. [6] and Goldgaber et al. [7] mapped the Aβ sequence to chromosome 21. Based on these reports Kang et al. [8] published a full sequence of the amyloid precursor protein (APP). APP is a highly conserved, single transmembrane protein which is a member of a family of proteins found in all animal species from *C. elegans* to man. These proteins, including Amyloid Precursor Like Proteins (APLP) 1 and 2, are growth factors that are necessary for normal neuronal development [9]. But it is unlikely that the Aβ portion of the APP sequence serves any important physiological function since it is not found in the APP of most species. The APLP proteins rather than APP are more generally found in various animal species. For example, *Drosophila* expresses a full-length member of the APP family protein which is required for normal neuronal development [10].

Furthermore, knocking out APP alone leads to some loss of cognitive skills, but is not lethal [11]. These mild deficits are well tolerated since other members of the APP family can promote neuronal growth. Knocking out any two of the three members of the APP family was lethal [11].

### The Metabolism of APP.

In order to understand the current studies in Alzheimer’s disease, it is first necessary to understand the origin of Aβ. As indicate below our studies indicate that it is produced during the posttranslational processing in the endoplasmic reticulum (ER) of a single transmembrane protein the amyloid precursor protein (APP) [13]. Figure 1

In vertebrate species the full-length APP is converted to one of two active soluble forms, sαAPP and sβAPP through cleavage by one of two proteases, α-secretase or β-secretase. The cleavage site for the α-secretase is 16 amino acids down from the cleavage site of the β-secretase. Since the sequence for a third of Aβ is between these two sites, the full-length peptide is only formed when APP is cleaved by the β-secretase to give sβAPP. The β-secretase is a membrane bound asparyl protease, BACE1 (Haas et al. 2012). If the cognitive decline is due to the neurotoxicity of Aβ, then inhibiting BACE1 would seem to be a logical treatment for Alzheimer’s disease. In support the role of β-secretase activity in the development of the cognitive decline...
The familial forms of early onset Alzheimer's disease are associated with mutations in three proteins: APP, presenilin1 and presenilin2. The first of these, APP, which is encoded on chromosome 21 was found to be associated with 29 variants of familial Alzheimer's disease [25]. Furthermore, linkage genetic studies demonstrated that the early onset disease resulted from a variety of mutations in APP. Two other proteins were also associated with early onset disease, presenilin 1 (psen1) on chromosome 14 and presenilin 2 (psen2) on chromosome 1. The presenilins are components of the protease, γ-secretase. This a plasma membrane bound protease that is involved in the excision of the membrane portion of Aβ. It is also involved in cell signalling. In particular the γ-secretase is involved in notch signal. Notch is a membrane-bound receptor for integrins [26]. When activated it activates the γ-secretase which clips off the intracellular portion of the receptor that then acts as a transcription factor.

The γ-secretase consists of four components including the two presenilins and two structural proteins [20]. The presenilins are the protease catalytic sites. The associated proteins are nicastrin and APH-1. Subsequent genetic studies have identified a large number of variants in each of the mutant genes. There have been reported to be 166 of the psen1 gene and ten in the psen2 gene [27]. The development of small molecule inhibitors of γ-secretase blocked the cognitive decline in transgenic mice, but not in patients with late onset disease. In fact, in one trial the experimental group exhibited decreased cognitive function compared to the placebo group [28]. This finding is not surprising since this protease catalyzes the activation of Notch an important plasma membrane receptor and inhibitors of Notch have been studied as potential cancer therapeutic agents.

Over half the cases of early onset disease are due to the various mutations in the psen1 gene. These genes are dominant negatives. Hence a haploid mutant lead to the development of an early version of the familial form of Alzheimer's disease.

In the case of the three genes associated with the early onset disease all of them lead to an increase production of Aβ. As a result of this association Selkoe [29] has proposed that the dementia found in Alzheimer's disease was due to the neurotoxicity of the Aβ. He proposed that the deposits and soluble aggregates of this peptide were either directly toxic or led to an inflammatory response that, in either case, caused a progressive neuronal injury which leads to the cognitive decline. It is not clear why these three mutations lead to an increased production of APP.

Several workers have identified a number of other problems with the β-amyloid model. These have included a poor correlation between plaque burden and the development of dementia; the most dramatic issue concerning the toxicity of Aβ [30,31] In spite of the questions which have been raised the majority of the workers in the field still accept the Aβ paradigm. They have raised the question of whether Aβ toxicity is due to the deposits or soluble aggregates. Irrespective of which of the forms of Aβ is toxic they still claim that transgenic mice are valid models for the disease.

The most widely accepted suggestion for the failure of monoclonal antibodies which clear plaque but fail to decrease cognitive decline is because these trials were initiated after the patients' disease had progressed to a point where they could no longer benefit from therapy [32-36]. In line with this concept Biogen and Roche are currently conducting clinical trials with monoclonal antibodies to examine the question of whether beginning therapy early in the disease can improve outcomes.

The Role of Apolipoprotein E in Late Onset Disease

Another area of concern is that until recently the only genetic variant which has been consistently associated with an increased incidence and earlier onset of late onset cognitive decline is the presence of at least one allele of apoE [37,38]. The odds ratios for the development of dementia for the various genotypes range from 0.6 for the E3/E3 to 1.9 for the E4/E4 genotype [39]. Yet, the estimated attributable risk ranges from 0 to 95%, with the usual estimates being between 30% and 50% [37,40,41]. This increased risk differs markedly among various populations [39,42].

Since apoE is a component of all of the major plasma lipoprotein fractions and the apoE, genotype has also been associated with an increased incidence of cardiovascular disease, one proposal for this effect of the apoE gene-isotype has been that the increased incidence of dementia is due to increased cerebral vascular disease [43]. Other suggestions such as decreased clearance of plaque [44] or binding of apoE to Aβ [45] would seem to be unlikely because they do not indicate how this leads to the initial accumulation of the deposits. Furthermore, apoE does not appear to be a primary binding protein for the naked peptide in the CSF, but rather it has been reported in immunoprecipitation studies that Aβ added to CSF primarily binds to apolipoprotein J, also known as clusterin [46]. It is of interest that in several Genome Wide Association Studies (GWAS) SNP's in this gene appear to be another gene, along with apoE4, that has been frequently associated with an increased incidence of Alzheimer's disease [27]. Furthermore, in a recent publication from the Rotterdam study, it has been reported that clusterin levels are higher in individuals with Alzheimer's disease than in normal subjects [47] suggesting that this binding protein may be induced by the presence of the naked Aβ-peptide and serves to blunt any possible Aβ toxicity.

Yet, new data from Roses’ laboratory has suggested an alternative mechanism whereby the gene load of apoE4 could affect the development of dementia [48]. In a GWAS they reported that the best correlation between the early onset of the late onset dementia was not with the apoE allele, but rather with a tightly linked gene for TOMM40/TOM40 which is about 30 kb upstream from the apoE gene. In these studies, it was found that the incidence and time of onset of the cognitive decline was associated with the length of poly-T runs in intron 6. TOMM40/TOM40 is a component of a protein transport pore in the outer membrane of the mitochondria (Humphries et al. 2005). The bulk of mitochondrial proteins are synthesized in the cytosol and transported into the mitochondria through this pore.

Valla et al. [49] reported that mitochondria from individuals with the apoE4 allele have decreased cytochrome oxidase activity, suggesting that defects in this pore lead to compromised mitochondrial function. This lower activity may be due to decrease content of TOMM40/TOM40. The lower TOMM40/TOM40 content is most likely not due to a decrease in the life span of the mRNA since these poly-T's do not code for any miRNA's which might regulate its half-life. It is possible that the poly-T runs could lead to alternative splicing of the mRNAs which in turn would lead to truncation of the TOMM40/TOM40 protein and possibly decreased function. Alternatively, it could just decrease the transcription of the gene.
In G W AS studies, another gene, clusterin, which appears to be associated with late onset disease. This is a plasma chaperone. Low levels of this protein in blood are associated with early onset of late onset Alzheimer's disease [50-52]. Low levels of clusterin is a potential biomarker for Late Onset Alzheimer's disease.

This raises an important issue. In view of the recent finding that the treatment of early disease with monoclonal antibodies to Aβ slows progression of the cognitive skills several pharmaceutical firms are involve in clinical trials to ascertain whether anti-Aβ monoclonal antibodies will slow the cognitive decline in the treatment of patients with relatively mild cognitive impairment. A major concern in testing these antibodies is to identify patients who are likely to benefit from this therapy. Currently potential patients are identified by radioactive Aβ brain scans this is both expensive and has a low sensitivity. A more efficient method would be to identify a biomarker that might be less expensive and more sensitive. The recent observational findings would suggest that clusterin may be a more robust biomarker that might improve identification of potential patients who are at an early stage of the decline. This is not consistent with the findings of the Rotterdam study [47]. One approach has been to look for genetic markers that could classify patients who could benefit from antibody therapy. Finally, Lilly has recently announced that it has halted its phase III trial of the Aβ monoclonal solenazumab for a lack of efficacy (Doody et al. 2014).

In line with this approach four large QWAS studies of patients with late onset Alzheimer's disease have identified several genes that may be associated with an increased incidence of the disease. Yet, most of the genes which were reported to have possible SNPs in all of these trials the only two that were identified in more than one trial were apoE and clusterin. Early purification and mass spectrographic studies reported by Ghiso et al. [46] found that when Aβ was added to the CSF, it is specifically and tightly bound to clusterin. Since then a number of groups have examined the association between clusterin and Aβ. Furthermore Jongbloed [51] reported that low plasma levels of clusterin were associated with an increased incidence of late onset cognitive decline. This relationship between clusterin levels and cognitive decline is consistent with the Aβ model since clusterin inhibits the nucleation of soluble Aβ to form aggregates. The high affinity of clusterin for Aβ has been reported by several groups in more recent studies.

A surprising finding is that clusterin infused into the right ventricle of the brain prevented traumatic brain injury in mice [53].

As an aside it is of interest that Muller et al [54] were able to obtain the blocks from Alzheimer's original patient and extract her DNA. They reported a single mutation in the psenlin gene. Rupp et al. [55] attempted to replicate their finding, but reported that the psenlin gene was normal.

**Neurofibrillary Tangles**

The other deposits found in the brains of Alzheimer's patients are tangles. These are aggregates of a neuronal cytoskeleton protein, tau. [56-58]. The protein kinase GSK3β phosphorylates several serines and threonines on tau. The hyperphosphorylated tau then aggregates to form intracellular deposits termed tangles. Knocking down GSK3β reduces plaque formation [57]. Furthermore, activation of this kinase increases the levels of BRACE1 [58] and thereby increasing the level of Aβ production. Of interest studies by Professor Wischik at the University of Edinburgh have suggested that the urinary analgesic, methylene blue clears tangles in the transgenic mice. In an early phase I trial, he found that a low dosage of this agent completely prevented cognitive decline. But unfortunately, in an unpublished phase III trial in patients it has reported that the drug had no effect on the progression of Alzheimer's disease [59].

**Our Findings on the Biochemistry of Aβ.**

The deposition of plaque in the elderly presents an interesting question: If everyone produces Aβ, why are deposits only seen in the elderly? Aβ is the major component of the plaque observed in the brains of patients with Alzheimer's disease. The conundrum is that although Aβ is produced in everyone during the posttranslational processing in the endoplasmic reticulum (ER) the Aβ, deposits are only observed in the brains of the elderly.

**Immunoblotting of the Aβ Complex in the CSF**

Our work suggests that normals have a carrier protein(s) that keeps Aβ in solution. Based on immunoblotting studies of cerebrospinal fluid (CSF) from cognitively intact subjects, we find that the bulk of the Aβ is N-glycosylated and bound to two ER chaperones, ERP57 and calreticulin, suggesting that these may be the carrier proteins which prevent aggregation of Aβ and that the deposits are due to faulty ER posttranslational processing of APP with the failure to form the soluble complex. If, as is likely, membrane protein synthesis is similarly affected, it could explain the neuronal dysfunction characteristic of Alzheimer's disease.

Since a portion of the CSF is derived from the extracellular fluid, it is the best approach to determine whether there is increased Aβ production in Alzheimer's patients is to determine their CSF concentrations. Yet, when this has been done, it has been found that the total concentrations of Aβ in the CSF of late onset Alzheimer's patients was equal to or less than that found in normals [60] indicating that factors other than the rate of Aβ production lead to plaque formation. This is unlike early onset familial disease in which the Aβ in the CSF is actually elevated.

For example, one factor could be that Alzheimer's patients have a nucleation factor which enhances plaque deposition. This may be the basis for the increased plaque seen in individuals with at least one apoE allele [61] since under oxidizing conditions apoE enhances Aβ precipitation more than do the other two isofoms.

Since APP undergoes posttranslational processing in the ER, we
posited that Aβ could be bound to one or more ER chaperones. To examine this possibility, we examined CSF samples from a number of cognitively normal patients obtained at the time of surgery for prostate problems.

On western blotting we found that all the Aβ in the CSF was found in a band at 62 Kda (Figure 3b). Similarly, one ER chaperone ERp57 appeared as a doublet with the upper band that also had an Mr=62 Kda which we presumed to be an ERp57-Aβ complex (Figure 3a)

We previously observed this same doublet when we initially purified ERp57 from rat liver [62]. Unfortunately, we never went on to separate the two bands, obviously even though Aβ and ERp57 have similar Mr’s doesn’t prove that they are present as a complex. Hence, we went on to immunopurify the complex and found that the two proteins copuriify the identity of the abetas band in the CSF has been confirmed with three monoclonal antibodies, Mab1-17, Mab1-42a and Mab1-42h, and our chicken antibody. On the other hand, we could not detect the complex when we used the monoclonal antibodies, Mab17-24, (4G8) even with very high concentrations of antibody (≥ 1 mg/mL) suggesting that the binding of Mab17-24 to the abetas was sterically hindered. This hindrance may be due to either N-glycosylation at asparagine 27 or O-glycosylation at serine 26 of the abetas. The failure of Mab17-24 to detect the abetas in the complex is consistent with the observations first reported from High’s laboratory indicating that ERp57 only binds to N-glycosylated proteins [63]. Unfortunately, for reasons which are unclear at the present time, N- and O-glycosidases (H- and G-) did not hydrolyze the complex suggesting that these enzymes do not have access to the asparagine27. On the other hand, as noted below, the complex could be hydrolyzed in base.

In light of the failure of others to report that the bulk of the abetas appears to be bound to a carrier protein, it has been repeatedly suggested that our observations are an artifact of the antibodies we have used. Although this is a legitimate concern, we feel that it is very unlikely that our results are due to cross-reactivity with other proteins. In response to this criticism we have employed a number of antibodies, many of which have been widely used by other workers in published studies. In particular, it should be noted that it is extremely unlikely that the reaction of the monoclonal antibody, Mab1-17, is due to an artifact of the assay procedure. This antibody is reactive to an epitope in the first 17 amino acids of the abetas. In a search of the NCBI protein data base, we have found that the sequence of this segment of the peptide is unique and highly conserved in vertebrates. Hence, there is very little likelihood that this antibody is nonspecifically cross-reacting with other proteins. Furthermore, as a result of its generally recognized specificity, this antibody has been widely used by many workers to identify the abetas in samples containing a heterogeneous collection of proteins, such as those found in plaque. If there were a problem with cross-reactivity with this antibody, it would raise the question of the validity of these other studies. Similarly, none of the antibodies we have employed for the detection of either the abetas or ERp57 reacted with either keratin, a major contaminant of tissue preparations, or clusterin, a previously identified carrier protein [12,13].

Work from High’s laboratory [63] has suggested that ERp57 only binds to proteins at N-glycosylation sites along with another ER chaperone, calrectulin. And indeed, when we examined the complex by a western blot run on a native gel without DTT, the Aβ, ERp57 and calrectulin all had Mrs of 130 daltons (Fig.5). This is the theoretical combined sum of the molecular weights of the three proteins [13].

One question which arises is whether the ERp57 is covalently bound to the Aβ? The standard procedure for purifying Aβ from plaque is to solubilize it in either concentrated formic or trifluoroacetic acids [64]. Yet when CSF was treated with either acid employing a standard protocol, the complex remained intact (Figure 6: channels 3,4). This observation would suggest that the ERp57 and Aβ are covalently bound. Similarly, two chaotropic agents, 6 M urea and guanidine isothiocyanate had no effect on the complex (Figure 6; channels 5, 6). On the other hand, the complex partially bound to a polyboronate column (Channel 7) indicating that it is glycosylated. It was released from the column with a glycine buffer (Channel 8). When the complex was exposed to a mild alkaline buffer overnight, it was hydrolyzed and the Aβ was seen at a Mr of around 10 Kda suggesting it was partially hydrolyzed and the resulting naked peptide aggregated to form oligomers (Figure 6; Channel 9).

Our findings would suggest that plaque formation is due to either a decline in the ER chaperone content or in the activity of the N-glycosylation pathway, the only glycosylation pathway in the ER. Both mechanisms seem possible. In a separate study we found that the hepatic content of ERp57 showed ca. 50% decline with age. Figure 7
The strange thing about this study is that the ERp57 and several other ER chaperones showed peaks in January and July. This had nothing to do with the temperature of the animal quarters, since the room was windowless and the temperature was held to within 1°C year-round. Most likely this is an epigenetic phenomenon that was established when the animals were in the wild and had to survive the harsh environmental conditions.

Table 1: The effect of age on some ER chaperones.

<table>
<thead>
<tr>
<th>Chaperone</th>
<th>Peak mg/g-liver</th>
<th>Concentration @874 days mg/g-liver</th>
<th>Constitutive</th>
<th>Show Cyclic Variation</th>
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</thead>
<tbody>
<tr>
<td>Bip</td>
<td>80.0</td>
<td>48.5</td>
<td>39</td>
<td>Yes</td>
</tr>
<tr>
<td>Calnexin</td>
<td>57.4</td>
<td>40.5</td>
<td>70</td>
<td>No</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>76</td>
<td>4.8</td>
<td>8</td>
<td>No</td>
</tr>
<tr>
<td>ERp55</td>
<td>34.8</td>
<td>13.1</td>
<td>51</td>
<td>Yes</td>
</tr>
<tr>
<td>ERp57</td>
<td>15.4</td>
<td>8.7</td>
<td>39</td>
<td>Yes</td>
</tr>
<tr>
<td>ERp72</td>
<td>144</td>
<td>100</td>
<td>30</td>
<td>No</td>
</tr>
<tr>
<td>Total</td>
<td>336.2</td>
<td>215.1</td>
<td>37</td>
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</table>

The ERp57-Aβ complex appears to be N-glycosylated at ASN27. In line with this hypothesis all the antibodies to Aβ that we used only Mab17-24 (with an epitope of 17-24) could not detect the Aβ, even with very high concentrations of antibody (≥ 1 mg/mL) suggesting that the binding of Mab17-24 to the Aβ was sterically hindered. This hindrance may be due to either N-glycosylation at ASN27 or O-glycosylation at serine 26 of the Aβ. The failure of Mab17-24 to detect the Aβ in the complex is consistent with the observations first reported from High’s laboratory indicating that ERp57 only binds to N-glycosylated proteins [63].

Unfortunately, for reasons which are unclear at the present time, N- and O-glycosidases (H- and G-) did not hydrolyze the complex suggesting that after passage of the complex through the golgi these enzymes do not have access to the ASN27. On the other hand, as noted above, the complex could be hydrolyzed in base. Finally, in support of the existence of this complex, we have in preliminary studies isolated and tentatively identified it from tissue preparations (Erickson et al. unpublished data). One problem with this paradigm is that the conical amino acid sequence for N-glycosylation is ASM- while the Aβ sequence is SER-ASN. This sequence may also guide the glycosylation pathway.

**Figure 5:** Immunoblot of CSF run on a native Gel. Channel 1 - anti-calreticulin; channel 2 - anti-Aβ; channel 3 - anti-ERp57. The bands were detected with the alkaline phosphatase reaction.

**Figure 6:** Immunoblots with rabbit polyclonal antibodies to Aβ (1282) of normal human CSF after treatments to dissociate the Aβ-ERp57 complex. Channel 1 - molecular weight markers; channel 2 - untreated CSF; channel 3 - CSF treated with 70% formic acid; channel 4 - CSF treated with 80% trifluoroacetic acid; channel 5 - CSF treated with 6 M guanidine isothiocyanate; channel 6 - CSF treated with 6 M urea; Channel 7 - CSF proteins which did not bind to a boronate column; Channel 8 - CSF proteins which bound to a boronate column eluted with pH 9 glycine buffer; channel 9 - treatment of the complex with glycine buffer, pH 9.0 Channel 9 – complex stored over night in pH 9 glycine buffer at 4°C.

**Figure 7:** The effect of age on the ERp57 content of rat liver. (Erickson et al. 2006).
The N-glycosylation Pathway:

The first step in the N-glycosylation pathway is the synthesis of a 15-saccharide carbohydrate complex attached to a high molecular weight lipid cofactor, dolichol [65-67] (Fig. 8). The construction of this carbohydrate complex is catalyzed by a series of monosaccharide transferases. The first sugar added to the dolichol phosphate is N-acetylglucosamine (Fig. 8). The strange thing about dolichol plus dolichol phosphate is that a number of laboratories have found that in the liver it increases 5-10-fold with age [68]. Since there is no increase in dolichol synthesis, it would appear that the accumulation of dolichol is due to a metabolic block at the level of the addition of the first sugar to the dolichol phosphate (Fig. 8). The addition of this first sugar to the dolichol is catalyzed by a specific monosaccharide transferase, MST, ALG7. These data suggest that this MST declines with age and that plaque formation is due to the inability of the ER to synthesize this complex and thereby releasing the naked Aβ. This pathway is critical for the synthesis of most membrane and secretory proteins.

If proteins are not correctly folded they are then degraded by the ER associated degradation (ERAD) pathway [69]. The failure to properly fold the nascent protein also activates the unfolded protein response (UPR) [70]. The UPR is controlled by three receptors in the ER membrane. In order to prevent clogging of the lumen of the ER with aggregates, these receptors signal the nucleus to turn off the transcription of proteins that are not critical for cell survival.

It has been found by a number of investigators that in plaque and Aβ present only as the naked peptide [71]. In view of our findings that Aβ in the CSF is only present as a complex would suggest that plaque accumulation in the elderly is due to a decreased capacity of the ER to catalyze the posttranslational processing of nascent APP. Since 40% of the proteins in the cell, including many of the synaptic membrane proteins that are required for a functioning memory, are APP, processed in the ER, these observations would suggest that the cognitive decline seen in the elderly is due in part to a decreased capacity of the ER to catalyze the processing of the key secretory and plasma membrane proteins that are necessary for a functioning memory. Compounding this problem, the decreased activity of the ER also leads to a decline in the golgi apparatus which is necessary for the final modifications of proteins that are processed in the ER [72]. An interesting aside is that in the elderly, the decreased activity of the ER may also be due to decreases in gene transcription. On the other hand, a number of studies have suggested that the decline may also be due to changes in the activity of the N-glycosylation pathway. This concept is based on the effect of age on the content of dolichol in various tissues, a necessary cofactor for N-glycosylation.

The initial step in the N-glycosylation pathway is the synthesis of an oligosaccharide bound to dolichol phosphate [65-67] (Fig. 8). Dolichol is a high molecular weight terpine which serves as a cofactor for the cytosolic synthesis of the oligosaccharide. Once the complex is fully synthesized, it is transferred to the lumen of the ER and then is attached to an ξ-amino group of asparagine by an oligosaccharide transferase (OST) [66]. The addition of the individual sugars to the carbohydrate complex is catalyzed by a family of specific monosaccharide transferases (MST) [65-67]. The first sugars added to the dolichol phosphate are a pair of N-acetylglucosamines, followed by nine mannosides and three glucoseoses. A number of studies have shown that as animals age there is a 5-10-fold increase in the cellular content of both dolichol and dolichol phosphate [68]. Since there is no increase in the synthesis of dolichol, this finding is consistent with a blockage of the addition of the first N-acetylglucosamine. And indeed, it has been reported that the activity of ALG7, the MST that adds the first N-acetylglucosamine to the dolichol phosphate, does decline with age [76].

In summary our data along with data from other laboratories suggest that the aging process is associated with a decline in the capacity of the ER to catalyze the posttranslational processing of nascent proteins, including most membrane and secretory proteins that are needed for a functioning memory. This paradigm would imply that the cognitive decline is not due to the toxicity of Aβ, but rather to a decline in the synthesis of the synaptic membrane proteins that are necessary for a functioning memory [13].

The ER and the Mitochondria: The effect of aging on Mitochondrial Function:

It is well known that as animal age their mitochondria acquire structural defects [77]. The damaged mitochondria are cleared from the cell by autophagy. Many of the proteins which make up the...
Autophagic vacuoles are processed in the ER and golgi [78]. Hence, with a decline in ER function there is also a decline in autophagy. Such declines have been associated with the onset of some age related neurodegenerative diseases [79].

Second, mitochondria are directly bound to the ER through the mitochondrial associated membrane (MAM) [80-82]. This structure serves three, major functions. The first is to transport critical phospholipids from the ER, where they are synthesized, into the mitochondria where they serve vital roles in the maintenance of the structure and function of the mitochondria. Further, it regulates Ca²⁺ homeostasis in the mitochondria and thereby oxidative phosphorylation [83-90].

A final role for the ER in maintaining mitochondrial structure and function is the control of mitochondrial fission and fusion. As defective mitochondria are cleared by autophagy, they are replaced through a process involving both mitochondrial fission and fusion [91-94]. Recent studies have indicated that several proteins in mitochondrial associated membrane (MAM) play major roles in both of these processes [80] (Friedman et al. 2011). In particular two critical proteins which tether the ER to the mitochondrial outer membrane are mitofusin 1 and 2 (mfn1 & 2) [80] (Fig. 9).

These are transmembrane proteins that are located in both the ER membrane and the outer membrane of the mitochondria. Both mfn1 and 2 undergo posttranslational processing in the ER. Hence, a decline in the capacity of the ER to catalyze their posttranslational processing would be expected to have profound effects on mitochondrial structure and function [95-99]. Such declines in ER function could compromise the ability of the cell to maintain metabolically active mitochondria. Together these findings suggest that one aspect of the decline in neuronal function seen with age is a decline in the capacity of the compromised mitochondria to maintain normal ATP production resulting from declines in the function of the ER.

It should be noted that even those individuals who have only the favorable forms apoE, apoE₂, and apoE₃, are still at risk for the development of Alzheimer's disease [40,41]. Other factors which have been proposed to cause the dementia, such as oxidative stress, inflammation, heavy metals, hyperphosphorylation of tau leading to tangles and advanced glycation end products, may enhance the progression of the disease, but I would contend that the decline in the synthesis of synaptic proteins is still the primary underlying defect that leads to the cognitive decline seen in the elderly.

**Why is there a loss of Myelin with Age?**

Finally, one well known clinical phenomenon is that it has been routinely observed in imaging studies that there is a decline in the volume of myelin with age. Myelin is associated with an onion like structure of the plasma membranes of oligodendrocytes in the brain and Schwann cells in the periphery [100-102].

There is no obvious connection between the deposition of plaque and the decreased volume of myelin seen in the elderly. This loss is seen even in areas which do not show plaque deposits. An alternative model for this decline is that it is due to the reduced capacity of the ER and golgi to process the plasma membrane proteins that are needed to maintain this structure [103]. And indeed, Jung et al. (2011) found that knocking out ERP57 and another ER chaperone, calnexin, led to faulty folding of critical myelin proteins. Since the ER content of both of these chaperones decline with age [13] (Table 1), this loss could account for the decrease in myelin seen in the elderly.

**Conclusions:**

In summary, published data suggest that the dementia seen in the elderly is not simply due to the neurotoxicity of the Aβ in the extracellular space of the cerebral cortex. The failure of the phase III clinical trials of the monoclonal antibodies bapineuzumab from Elan/J&J/Pfizer and solanezumab from Lilly, both of which have been shown to clear plaque, suggests that Aβ has does not have a major...
role in the cognitive decline seen in late onset Alzheimer’s disease [32-34]. A similar failure was seen in a vaccine trial [104]. These failures make it difficult to continue to accept the possible major role of the neurotoxicity of Aβ in the etiology of the dementia associated with Alzheimer’s disease [32-34]. These failures would suggest that we should develop new cellular and animal’s models in the search for effective therapeutic agents.

On the other hand, it is quite possible that the antibodies to Aβ may prove to be effective in individuals with Down’s syndrome or the familial forms of Alzheimer’s disease. In these conditions the dementia may be due to the excess production of sβAPP leading to continued apoptosis of neurons through Death Receptor 6 [105]. Although asAPP appears to be present at much lower concentrations than βsAPP, it is thought to be active at these concentrations (Herzog et al. 2004). Since the 17 carboxy terminal amino acids of asAPP are the same as the amino terminus of Aβ any antibody which is directed to an epitope in this region of Aβ will also react with and possibly clear asAPP. This could therefore prevent the continued apoptosis of neurons and the progression of the dementia.

Proposed Future Directions: New Animals and Cellular Models for Drug Discovery:

The total failure of the pharmaceutical industry to develop robust therapies for the treatment of late onset disease raises the question of whether they are using the wrong animal model to screen for potential drugs? Many government agencies and pharmaceutical firms have large libraries of compounds which can be screened for their potential activity in enhancing or blocking the specific pathways which have been identified as being important in a disease process. Once these pathways have been identified, it is necessary to develop a reporting system to facilitate the rapid identification of potentially effective therapeutic agents. In the case of aging in general and Alzheimer’s disease in particular, our basic biochemical studies have suggested that the physiological functional declines that are seen are due to a decrease in N-glycosylation and/or a decline in the chaperone content of the ER. As a result of these losses there is a decline in the capacity of the ER pathway to fold proteins into their active configurations. Hence, the logical approach is to identify agents which can enhance the production of the ER chaperones and the enzymes that are involved in the N-glycosylation pathway.

Classically workers have constructed pseudo genes in which the promoter region that regulates the transcription of the gene encoding the target protein is coupled to a reporter containing the genes for proteins such as luciferase, galactosidases or various fluorescent proteins. The usual fluorescent proteins are various derivatives of the green fluorescent protein (GFP) such as red (RFP), cyan (CFP), yellow (YFP) and mCherry fluorescent proteins. Each of these indicator proteins contain the coding for the protein sequence, the introns which are inserted between the exons and various control regions including the promoter region which precedes the first exon and the region which follows the last exon, the 3’untranslated region (3’-UTR). As a result, the activation of a gene and the translation of the mRNA is regulated by the RNA coded in sequences in all of these various regions of the gene.

Furthermore, in all eukaryotic cells the DNA is rapped around a set of nuclear proteins, the histones, to form chromatin. This compacts the DNA and makes it possible for a large amount of bulky DNA to fit within the nucleus. Hence, the first step is for the cell to unwind the portion of the DNA containing the relevant gene. This process is mediated through the activation of various enzymes which modify the histones by the addition or removal of various modifications, such as phosphate, acetyl or methyl groups. These modifications are catalyzed by a set of enzymes in the nucleus which in turn are controlled by a number of factors.

Once the DNA is freed from the histones the specific gene is activated by protein complexes which are termed transcription factors which bind to gene-specific sequences on the DNA. The transcription factors are mobilized by a variety of reactions, most of which occur in the cytosol. The activated transcription factor complex is then translocated into the nucleus and bind to the appropriate sites on the DNA activating or repressing the gene. Furthermore, there are enzymes that bind the DNA, unwind its secondary and tertiary structure and separate the activated gene from its bound complementary strand of DNA. This then initiates the transcription of the gene to form a RNA complimentary copy of the exons and introns. The copy is then spliced to give the message RNA (mRNA) which then translocates into the cytosol where it binds to a set of particles, the ribosomes which catalyze the translation of the mRNA. Other factors bind the mRNA to initiate and inhibit translation of the message to form the gene product protein. After the message is translated a number of times, the mRNA is destroyed. The number of times that the message is translated is determined in part by sequences found in the 3’-UTR of the mRNA.

A further complication is that ironically, when introns were first discovered, they were thought to be “junk DNA.” That is they seemed to serve no obvious function, but a vast number of studies have clearly demonstrated that they play vital roles in the control of gene expression and hence protein synthesis. Similarly, the 3’-UTR region contains sequences which seem to be involved in the control of transcription. In view of the complexity of this process and the large number of sites which play a role in the control of transcription and translation, it would seem that the potentially most fruitful approach to identifying effective therapeutic agents would be to develop screening techniques which leave the gene and the associated control systems as much intact as is possible. Based on these considerations, the construction of pseudo genes containing only the promoter region does not permit the widest search for drugs affecting the various control points in the initiation and completion of the synthetic process.

I feel that the body of evidence indicates that a more likely paradigm for the etiology of the dementia associated with Alzheimer’s disease is due to declines in the chaperone content of the ER or the activity of the N-glycosylation pathway. This proposal would suggest new cellular models for high through-put drug screening in which the exons of ERp57, and other chaperones and the various MST’s are labeled with a fluorescent protein, such as GFP. The cells could then be incubated with the test compounds and screened in a microtiter plate reader for increased fluorescence compared to those incubated with the vehicle alone. Studies from my laboratory would suggest that a good initial candidate for such trials would be the insecticide, methoxychlor, since in toxicity studies we incidentally found that feeding low doses of this
agent specifically increased the ERp57 content of hepatic microsomes (Morrell et al. 2000).

Similarly, it would also be possible to create animal models based on this paradigm. In these studies, the animals could be transfected with a plasmid containing a conditional promoter and the sequence for small, antisense RNAs directed to the mRNAs of the chaperones or the MST’s. Since both the chaperone and MST knockouts are lethal mutants, for cognitive testing the transcript would have to be activated in mature animals by the administration of some agent such as doxycycline. The administration of antisense reagents directed to these various targets would cause problems with the psychomotor testing since the loss of function could be due to a loss of muscle mass.

These animal constructs would also serve as proof of principle model systems. For example, in validating the hypothesis that the loss of cognitive function is due to a decline in the posttranslational processing of proteins in the ER, the animals’ cognitive and motor skills can be tested by standard psychomotor procedures both before and after the antisense is activated. Similarly, it could also be used to determine whether the test proteins have a role in the loss of immunological function, cardiac function, kidney and insulin production and the decrease in other tissue functions seen with aging.

References