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Methods and models in neurodegenerative and systemic protein aggregation diseases

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1. ABSTRACT

Protein misfolding and aggregation are implicated in a wide range of increasingly prevalent human diseases ranging from dementia to diabetes. In this review we discuss the current experimental strategies that are being employed in the investigation of the pathogenesis of three important protein misfolding disorders. The first, Alzheimer's disease (AD), is the most prevalent neurodegenerative disease and is thought to be initiated by the aggregation of a natively unstructured peptide called amyloid beta (Abeta). We discuss methods for the characterization of the aggregation properties of Abeta *in vitro* and how the results of such experiments can be correlated with data from animal models of disease. We then consider another form of amyloidosis, where a systemic distribution of amyloid deposit is caused by aggregation and deposition of mutational variants of lysozyme. We describe how experiments *in vitro*, and more recently *in vivo*, have provided insights into the origins of this disease. Finally we outline the varied paradigms that have been employed in the study of the serpinopathies, and in particular, a dementia caused by neuroserpin polymerization.

2. INTRODUCTION

In the most highly developed countries the major challenges to human health over the coming decades will be disorders of affluence and old age, such as diabetes, Parkinson's disease and Alzheimer's disease. Indeed, as the population progressively ages we can reasonably expect a doubling in the prevalence of these common diseases by the middle of the century (1). While these diseases characteristically appear in the elderly, it now seems that they are not the inevitable consequence of "wear and tear", but instead they are the result of specific changes in the cellular metabolism of folded and unfolded proteins. To understand how best to intervene in disorders of protein misfolding and aggregation, it is vital that we investigate the molecular and cellular processes that result in disease, both at the molecular and at the organism level. In this chapter, we discuss some of the *in vitro* techniques and the cellular and animal modeling approaches that can be used to study the aggregation of a range of pathogenic proteins (reviewed in reference (2)). Some of these *in vitro* and *in vivo* assays, including their advantages and limitations, are listed in Tables 1a and 1b respectively. The specific proteins that we mention here are members of three

Table 1. *In vitro* and *in vivo* assays of protein aggregation behavior

Method	Advantages	Disadvantages
a. *In vitro* assays of protein aggregation behavior		
In situ thioflavin T (polypeptide aggregation occurs in the presence of the dye) (38).	Provides a measure of beta-rich aggregates. This is a convenient method for generating multiple observations over a time course.	Possible artifacts caused by the interaction of thioflavin T with aggregates.
Ex situ thioflavin T (aliquots are removed from the aggregation mixture and incubated with the dye) (28).	Provides a measure of beta-rich aggregates. There are fewer concerns about dye binding artifacts.	More labor intensive. Disturbance caused by removing aliquots may cause artifacts.
Circular Dichroism (CD) (33, 49-52).	Gives information about changes in secondary structure. No dye required.	The data is only semi-quantitative. Relatively large amounts of polypeptide are required.
Size Exclusion Chromatography (SEC) (34, 56, 58).	Allows separation of aggregates according to their sizes and shapes.	The equilibrium between aggregated species is disturbed during chromatography. The experiments are relatively lengthy.
Transmission Electron Microscopy (TEM) (61, 99).	Provides a measure of the structure and morphology of aggregates.	Quantitative analysis of images is difficult. The spectrum of aggregates that are imaged may not reflect the distribution in the sample.
Atomic Force Microscopy (AFM) (48).	Provides more quantitative data and higher resolution than TEM. AFM can be performed in solution.	More labor intensive and lower throughput than TEM.
Light scattering (99).	Gives information about the size distribution of aggregates in a solution. No dye required.	Light scattering has limited sensitivity and relatively high concentration of polypeptide is required. This method cannot differentiate between amorphous or ordered aggregation and cannot detect small aggregates in the presence of large ones.
Dynamic light scattering. (DLS) (32).	Gives information about the size distribution profile of smaller oligomeric assemblies.	This method is relatively labor intensive and requires high concentrations of polypeptide.
b. *In vivo* assays to probe effects of protein aggregation in *Drosophila*		
Longevity assay (28, 75).	Clear endpoint and robust statistical interpretation.	Lengthy experiments.
Climbing assay (28, 75, 157).	Locomotor deficits provide an early indication of neurodegeneration.	Manual assays are labor intensive however automation is possible.
Immunohistochemistry (75, 157).	Provides insights into the degree of accumulation and the location of protein aggregates.	Need protein- and aggregate- specific antibodies.
ELISA assay (81, 157).	Provides highly sensitive measures of protein levels.	Need protein- and aggregate- specific antibodies.
Genetic screening (81).	Allow the detection and dissection of the pathological pathways.	Lengthy experiments, particularly if recessive genetic modifiers are screened.

important classes of disease-related proteins; first we will discuss the amyloid-beta peptide as a representative of a group of proteins and peptides that are natively unfolded, while in disease they form microscopic, cytotoxic amyloid deposits within tissues, often in the nervous system. Secondly, we will discuss lysozyme as a representative of a group of natively folded proteins, that when misfolded form macroscopic amyloid deposits throughout the body. Thirdly we review neuroserpin, a protein that retains a near-native fold even after forming aggregates in the endoplasmic reticulum of neurons.

3. THE BETA-AMYLOID PEPTIDE AND ALZHEIMER'S DISEASE

In Alzheimer's disease, as in the majority of protein misfolding disorders, the characteristic protein deposits are composed of amyloid fibrils (2). It is remarkable that a wide range of polypeptides are able to adopt this well-defined fibrillar structure, characterized by a cross beta-structure in which beta-strands are oriented perpendicular to the direction of the fibril axis, and by the ability to display red-green birefringence when stained with Congo Red (3-7). There are two types of deposits that are characteristic of AD, the first is extracellular neuritic plaques composed of Abeta peptides and the second is intracellular tangles that are comprised of the protein tau. The plaques are composed of a number of isoforms of the Abeta peptide, each of which has distinct C-termini resulting from variations in the site of the endoproteolytic cleavage that generates the peptide from the amyloid precursor protein (8). The two major Abeta isoforms present in the human brain are $Abeta_{40}$ and $Abeta_{42}$ and the ratio of these peptides is clinically important; specifically an increased ratio of the longer $Abeta_{42}$ to $Abeta_{40}$ correlates with an increased risk of subsequently developing AD. Despite being secreted at much lower concentrations than $Abeta_{40}$, it is $Abeta_{42}$ that is the predominant component of plaques and is believed to be critically important in AD (9, 10).

The clinical importance of AD has encouraged a great deal of interest in elucidating the mechanism of Abeta aggregation and neurotoxicity. Although the mature amyloid fibrils, which comprise the neuritic plaques in AD, have historically been considered the pathogenic species, more recent research has focused interest on the prefibrillar oligomeric Abeta assemblies that have the potential to cause neuronal failure in AD (11, 12). Abeta amyloid fibrils formed *in vitro* have been shown to exhibit the same morphological properties as *ex vivo* amyloid fibrils and are also toxic to neurons (13-16). Therefore, synthetic Abeta peptides are widely used for characterization of the Abeta aggregation mechanism and its pathological consequences.

3.1. Preparation and handling of the Abeta peptide

The Abeta peptide has proven difficult to handle due to its high propensity to aggregate and therefore, many technical barriers must be overcome in preparing solutions of the peptide to allow well defined and reproducible biophysical and biological behaviors. Abeta peptides can be produced either by solid-phase synthesis, a technique in which amino acids are successively coupled to a growing chain attached to a polymer support, or by recombinant

Table 2. Methods for preparation of solutions of the Abeta peptide for *in vitro* aggregation studies

Method	Advantages/use	Disadvantages/limitations
High pH (NaOH) (21, 23).	This method of solubilizing the Abeta peptide avoids the isoelectric point and minimized aggregation.	Disruption of Abeta aggregates may be incomplete.
Low pH Trifluoroacetic acid (TFA) (162, 163).	This is an effective method for solubilizing the Abeta peptide and will completely disrupt aggregates. TFA is easily removed by lyophilization.	The pH transition from basic to neutral conditions will cross the isoelectric point of Abeta when the peptide is redissolved in neutral buffers which may result in re-aggregation of the peptide.
Dimethyl sulphoxide (DMSO) (21, 164).	DMSO effectively solubilizes the Abeta peptide.	High boiling point of DMSO means that it must be removed by buffer exchange techniques which may reduce the yield of the peptide.
Hexofluoro isopropanol (HFIP) (24).	This is an effective method for the disruption of aggregates. HFIP is also volatile and hence is easy to remove by centrifugation under vacuum.	HFIP is less effective than TFA for the initial solubilization of synthetic Abeta peptide.
Sonication (21, 162, 163).	An effective adjunct when disrupting aggregates.	Not sufficient to ensure complete disruption of aggregated peptide.
Centrifugation (21).	Often preceding size exclusion chromatography to remove or isolate larger aggregates.	The method needs to be optimized to minimize the contamination of the supernatant with aggregates.
Size exclusion chromatography (21).	Aggregates of different sizes and low molecular weight Abeta can be isolated.	The yield and final concentration of peptide may be low.
Filtration (21, 23).	Isolation of low molecular weight Abeta.	The yield of peptide may be low.

protein expression methods (17-20). As the aggregation propensity differs between Abeta variants, each variant may require specific modifications to the production process, consequently leading to problems in comparing the behavior of different variants or isoforms of a particular peptide. Depending on the batch of peptide or the method of preparation, there are likely to be variation in the aggregation state of the initial material, and the aim is to achieve protocols that can minimize this source of experimental variability.

A wide range of methods have been employed to prepare monomeric peptide stock solutions, ranging from the use of high and low pH, a variety of organic solvents and different biophysical techniques such as centrifugation, sonication, size exclusion chromatography and filtration (21). It is important to use a preparation method that will give a reproducibly homogenous initial state of the peptide, and that is free from pre-formed aggregates but also maintains a high yield of material. Notably, even in an apparently aggregate-free Abeta peptide solution, prepared at micromolar concentration, there is an equilibrium between monomeric and oligomeric structures that is not well understood. These species are described in the literature as low molecular weight Abeta aggregates (21, 22). In our experience, no single method of peptide preparation yields material that is optimal for every biophysical technique; however, we describe here a range of preparation methods and their uses and limitations (summarized in Table 2).

Using high pH is advantageous for solubilizing Abeta peptides as the transition from basic to neutral conditions avoids passing through the isoelectric point of Abeta (pI $\approx$ 5.5) (23). However, high pH might not be sufficient to disaggregate fibrillar forms of Abeta and thus a second step, such as filtration or size exclusion chromatography, is often needed to produce high quality monomeric Abeta (21). Such an additional step will reduce the yield of peptide and may result in greater variability in the final concentration of the peptide solution, but is essential to obtain reproducible aggregation kinetics.

The use of hexafluoroisopropanol (HFIP) at low pH has also been shown to be successful in disrupting pre-formed Abeta fibrils (24). However, in our experience, synthetic Abeta peptides can not be efficiently solubilized using HFIP alone. Instead, we find the addition of trifluoroacetic acid followed by sonication and lyophilization is a good starting point for preparing synthetic Abeta peptides. After this step the peptides can readily be dissolved in HFIP, divided into appropriate aliquots and dried by rotary evaporation. The amount of peptide in each aliquot can then be determined by performing quantitative amino acid analysis at least on the first and the last aliquots to ensure that the peptide concentration remains constant in each sample. Using this preparation method, the resulting $Abeta_{40}$ peptide in solution gives rise to a typical "random coil" CD spectrum with a strong negative ellipticity reading at 200 nm (Figure 1A) (25, 26). Furthermore, only three bands, corresponding to monomer, dimer and trimer are visible in western blots (Figure 1B). This finding is consistent with the observation of SDS-stable Abeta dimers in solutions of $Abeta_{40}$ at nanomolar concentrations (27). Using this preparation method, we have been able to reproducibly characterize the aggregation behavior of a range of different Abeta peptide variants on a routine basis (28).

3.2. *In vitro* aggregation of the Abeta peptide

A number of spectroscopic methods including thioflavin T fluorescence (29-31), light scattering (32), circular dichroism (CD) spectroscopy (33), size exclusion chromatography (SEC) (34) and transmission electron microscopy (TEM) have been used, alone or in combination, to study fibril formation of Abeta peptides. Perhaps the most commonly used method is to follow the spectroscopic signal from thioflavin T, where a substantial increase in the fluorescence emission at 482 nm can be detected upon binding to fibrillar structures (35, 36). A typical thioflavin T fluorescence profile of Abeta aggregation is presented in Figure 2, in which $Abeta_{40}$ displays a sigmoidal, characteristically bipartite aggregation profile consisting of a lag and growth phase (37). From this curve the rate of aggregation (k), which is a measure of the efficiency of the aggregation reaction, can be extracted from the maximal slope of the sigmoidal curve, or equivalently by fitting the growth phase to an

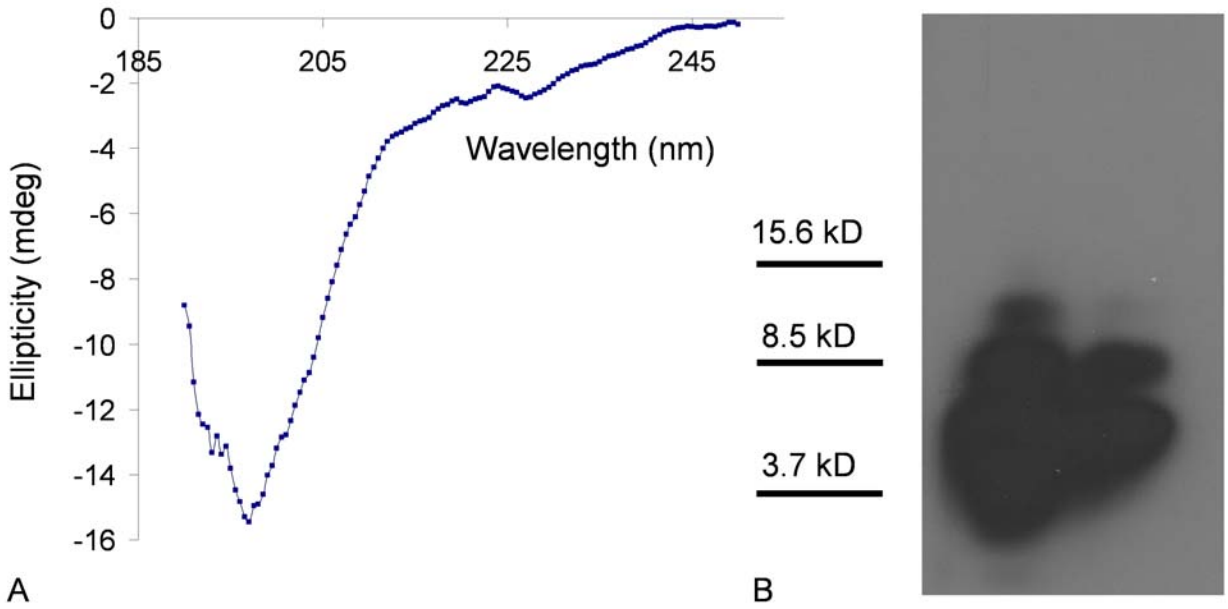

Figure 1. Characterization of Abeta$_{40}$ prepared at low pH. A) CD spectrum and B) western blot of Abeta$_{40}$. The synthetic peptide was dissolved in trifluoroacetic acid and sonicated for 30 seconds on ice. The trifluoroacetic acid was removed by lyophilization and the peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol, divided into aliquots and dried by rotary evaporation at room temperature.

exponential form: $y = q + Ae^{(-kt)}$ (38, 39). The length of the lag time (t^l) can also be determined by fitting the initial data to a straight line *a* and a tangent *b* to the steepest region of the growth curve; t^l is defined as the time point where the lines *a* and *b* intersect (Figure 2). The lag time is often considered to be the time required to form growth nuclei, and it has been assumed that its duration is determined only by the rate of nucleation events. However, recent measurements show that lag phases can occur even under conditions where nucleation is negligible and that signs of amyloid fibril growth can be detected during the lag phase (40). Therefore, it is likely that the lag time contains contributions both from the growth of aggregates, as well as from the *de novo* formation of new aggregates from soluble protein through nucleation.

Much interest has focused on identifying the elementary steps which underlie amyloid growth, by extracting information from bulk kinetic measurements and observing how changes in the microscopic rate constants affect the overall characteristics of the sigmoidal growth kinetics. It has become apparent that in addition to the processes of nucleation and elongation, fragmentation of existing fibrils is an essential factor for understanding amyloid growth (41-43). Indeed, as spontaneous nucleation is inherently a slow higher order reaction, the formation of new fibrils through fragmentation of existing structures could, in many cases, be a more effective source of new fibrils, the combination of elongation and fragmentation leading to exponential growth of the fibril population (43). For prions, it has been established that the most aggressive strains are those composed of fibrils with the greatest propensity to fragment and proliferate (44, 45); the finding that the Abeta peptide can also exist in different strain-analogous configurations *in vitro* and *in vivo* raises the question of whether fragmentation could have a similarly central role for the understanding of the aggregation of Abeta peptide and the different activities of the structures resulting from this process (46, 47). In this context, it is interesting to speculate that the less robust and less rigid nature of protofibrillar species, that precede the formation of fully formed amyloid fibrils, may allow them to fragment, proliferate and interact with cellular components more effectively than fully formed fibrils, thereby leading to increased pathogenicity (48).

The characteristics of a typical Abeta aggregation profile, probed by thioflavin T fluorescence, are strongly dependent on the design of the experiment and on the handling of the Abeta sample. *In situ* measurement of thioflavin T fluorescence has the advantage of leaving the Abeta sample undisturbed in the fluorometer throughout each experiment. But, the presence of thioflavin T in the reaction mixture may interfere with the aggregation process. In contrast, *ex situ* assays measure thioflavin T binding to aggregates in aliquots that are taken from a stock solution of polypeptide. Although *ex situ* assays avoid dye artifacts, the repeated sampling may in turn disturb the aggregation process. Another consideration when interpreting thioflavin T fluorescence data is that the intensity of the signal at the end of the aggregation process varies greatly between different Abeta aggregates, likely reflecting variations in the binding of thioflavin T to aggregates with different morphologies. For example, despite differing by only two C-terminal residues, the Abeta$_{40}$ and Abeta$_{42}$ peptides display profound differences

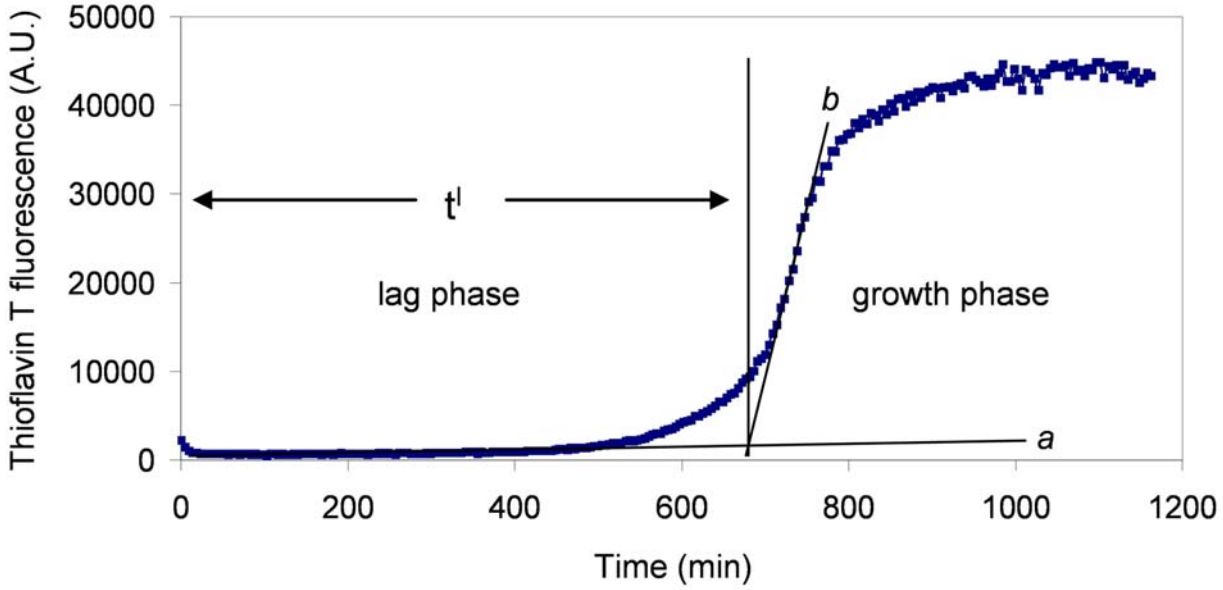

Figure 2. Monitoring aggregation of $Abeta_{40}$ using thioflavin T fluorescence spectroscopy. The samples contained 40 microliter of 40 micromolar $Abeta_{40}$ in 50 mM sodium-phosphate, pH 7.4 and 60 micromolar thioflavin T. The reaction mixture was incubated at 29 °C and data points were recorded every 4.7 minutes with 10 seconds of orbital shaking preceding the measurement using a FLUOstar OPTIMA reader (BMG) equipped with 440 nm excitation and 480 nm emission filters. The peptide was prepared as described in Figure 1.

in their aggregation behavior. The $Abeta_{42}$ peptide has a higher propensity to form protofibrils than $Abeta_{40}$ and while the end stage aggregation products of $Abeta_{40}$ are usually long well-ordered fibrils (Figure 3A), the $Abeta_{42}$ fibrils are commonly accompanied by smaller aggregated species (Figure 3B). In our experience, the aggregation products of $Abeta_{40}$ give a higher thioflavin T fluorescence signal than aggregates of $Abeta_{42}$, highlighting the differences in the respective aggregated structures and probably reflecting an enhanced ability of $Abeta_{40}$ aggregates to bind to thioflavin T as compared to $Abeta_{42}$.

Circular dichroism spectroscopy has been used in several studies to define the conformational states of the Abeta peptide and to monitor transitions in secondary structure from random coil to beta-sheet (49-52). A typical spectrum recorded for a beta-rich amyloid structure is shown in Figure 3C, displaying a minimum at 219 nm and a maximum at 196 nm. CD spectroscopy has the advantage that measurements of secondary structure during the aggregation process can be made without the need for reporter dyes such as thioflavin T. In general agreement with the thioflavin T measurements, kinetics of Abeta aggregation recorded by CD spectroscopy typically show a characteristic three-step sigmoid profile comprised of a lag phase, an exponential growth phase and a plateau phase (53-55). The CD approach has also been found to be useful for measuring the effects of inhibitors of Abeta aggregation (52).

Size exclusion chromatography is a technique that allows the separation of aggregates of different size and shape and has been used to isolate and characterize specific aggregated species such as *in vitro* generated Abeta-derived diffusible ligands (soluble oligomeric forms of Abeta, abbreviated ADDLs) which have been shown to be neurotoxic (56-57). This technique has also be used to monitor the transition of monomeric peptide into protofibrils and then subsequently into amyloid fibrils (58). However, during the chromatography step, which can take up to an hour to complete, the equilibrium between aggregated species will be disturbed and thus the final chromatogram might not reflect the true composition of aggregates in the reaction mixture.

Dynamic light scattering can provide information about the size distribution profile of smaller oligomeric assemblies. For example, in a detailed study using a range of biophysical techniques including photo-induced cross-linking of unmodified proteins and size exclusion chromatography in addition to dynamic light scattering, it was possible to show that while the $Abeta_{40}$ peptide exists in an equilibrium between monomeric, dimeric, trimeric and tetrameric forms, the $Abeta_{42}$ peptide preferentially forms pentameric or hexameric units (paranuclei) which seem to be the initial and minimal structural elements from which assembly of Abeta into fibrils takes place (32).

The increasing evidence that it is oligomeric, rather than fibrillar, aggregates of Abeta that cause toxicity in AD has prompted much interest in the visualization of these smaller aggregates. We have found that a combination of negative stained TEM imaging and atomic force microscopy (AFM) is optimal for this purpose;

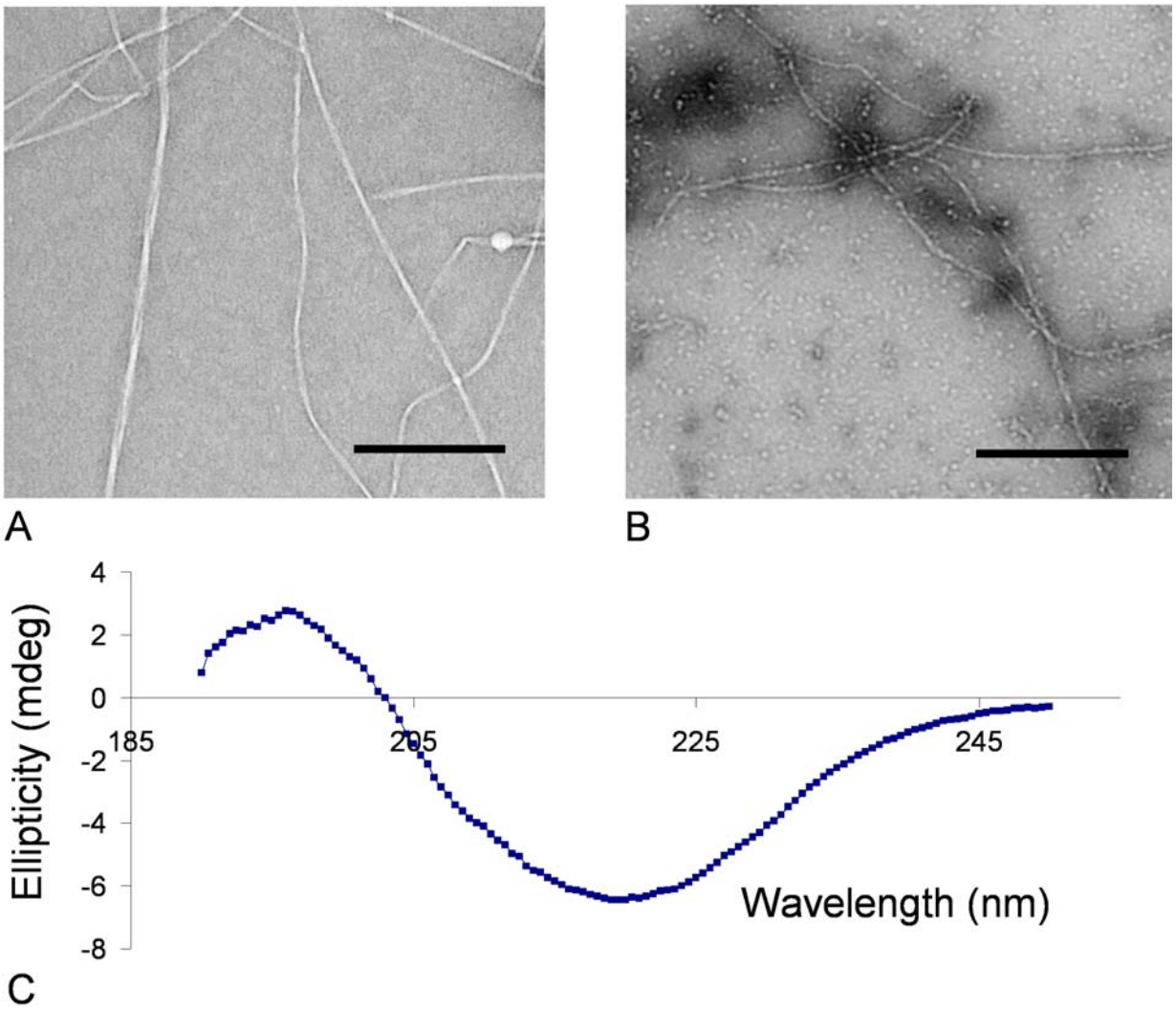

Figure 3. Characteristics of Abeta$_{40}$ and Abeta$_{42}$ aggregates. Aggregates of Abeta$_{40}$ (A) and Abeta$_{42}$ (B) imaged by TEM (scale bar = 200 nm). C) CD spectrum of Abeta$_{40}$ aggregates reveals the presence of beta-rich structures by a minimum at 219 nm and a maximum at 196 nm.

whereas AFM gives three-dimensional information about morphology at the nanometer scale and can be performed under physiological conditions (48), the electron microscope provides convenient data regarding the length and the width of the Abeta aggregates. Indeed, using such an approach, it has been found that the mutation E22G of Abeta$_{42}$, which causes familial, early onset AD, not only enhances the process of fibril formation but also accelerates the formation of oligomeric intermediate species (58, 59). Despite the transient nature of these neurotoxic aggregates (reviewed in reference (60)), these techniques show them to have a range of appearances including spheres, "beads on a string" and annular oligomers, where the latter have been suggested to have a potential role as specific membrane disrupting pores. A general scheme for Abeta aggregation is presented in Figure 4 where soluble Abeta monomers are converted to mature fibrils.

Structural studies of Abeta$_{40}$ and Abeta$_{42}$ fibrils, based on nuclear magnetic resonance and electron microscopy techniques, have revealed a protofilament, defined as the minimal fibrillar unit that is observed in the experiments, which is characterized by two stacked, in-register parallel beta-sheets that propagate along the fibril axis resulting in two-fold symmetry around the fibril growth axis (13, 14). The fibrillar core of Abeta$_{40}$ involves residues 12-24 (beta1) and residues 30-40 (beta2). In comparison, the fibrillar core of Abeta$_{42}$ was found to be slightly shifted towards the C-terminus, with beta1 and beta2 composed of residues 18-26 and residues 31-42 respectively. However, fibril morphology of the Abeta peptide is by no means homogeneous in nature. Recently, structural studies of Abeta$_{40}$ fibrils, with a characteristic periodically-twisted appearance observed by TEM, revealed a protofilament constructed of three cross-beta units that result in three-fold symmetry around the fibril growth axis (61). This implies that differences in the molecular structures of the fibrils underlie the diverse morphological nature of amyloid fibrils.

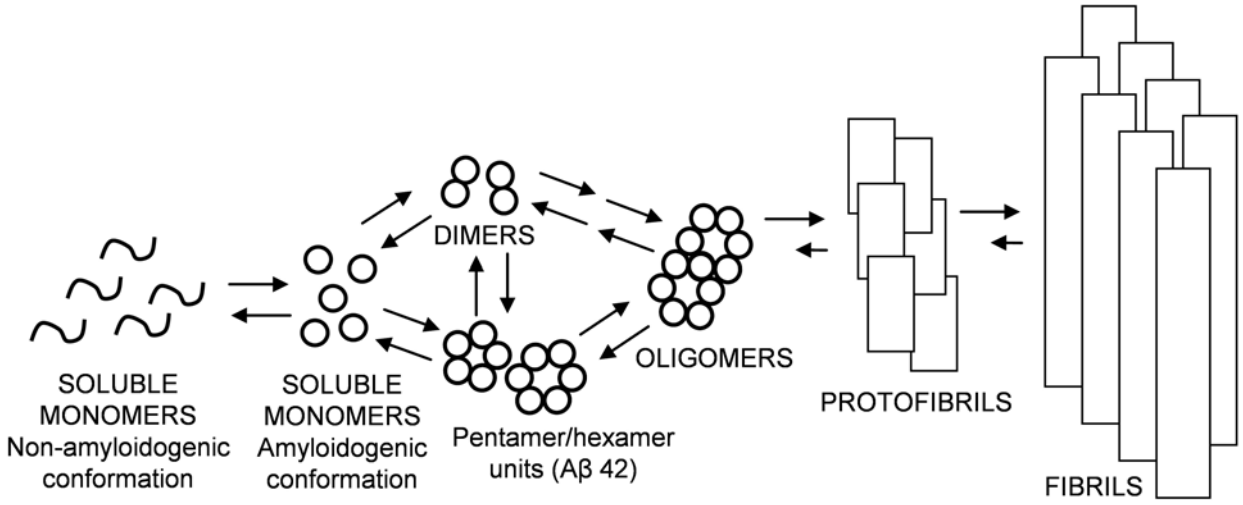

Figure 4. Schematic overview of Abeta aggregation. Soluble Abeta monomers that are non-amyloidogenic can adopt aggregation-competent conformations. In the case of $Abeta_{42}$ these monomers rapidly oligomerize into pentamer/hexamer units. Further aggregation of oligomeric assemblies results in the formation of protofibrils which are subsequently converted into mature fibrils. In addition, new fibrils can be formed through fragmentation and elongation of existing protofibrillar structures.

3.3. Abeta pathogenicity

To address the need to link the well-characterized biophysical processes of peptide aggregation to the biological pathways that mediate the neuronal dysfunction and death that characterize AD, a range of cell culture and animal models of AD have been established, each system having particular strengths and limitations.

Cell cultures are frequently used to examine the proteolytic processing of the amyloid precursor protein (APP), which generates the Abeta peptide (62-64). In particular, there has recently been great interest in determining the composition and activities of the proteolytic complexes that process APP. These activities named alpha, beta and gamma secretase, are of pharmaceutical interest because of their upstream location in the pathogenic cascade. Determining the proteolytic mechanisms of the secretases and their sub-cellular sites of action has been a particular achievement of the past five years. Building on this knowledge, Andersen and colleagues have shown that the subcellular trafficking of APP determines whether or not the protein comes into contact with the secretase machinery. In particular, a protein called SORL1 have been found to influence the risk of developing AD by determining whether APP is recycled to the plasma membrane after endocytosis or, alternatively, enters the amyloidogenic processing pathway (65-67). There are many other applications of cell biological models of AD, but crucially none give a robust model of Abeta toxicity. Although the generation of Abetą or Abeta-oligomers is taken as a surrogate endpoint for toxicity, specific data on Abeta toxicity can be obtained by the use of cell cultures, in which Abeta peptide is added, in relatively high concentrations, to the medium and then impairment in cellular function and viability is measured. An alternative and complimentary approach is to create whole-animal models of AD.

Traditionally, animal models of AD have been murine; indeed the best accepted models are exemplified by the triple transgenic mouse of LaFerla (68, 69). These mice express disease-associated variants of human APP, presenilin-1 and tau, and faithfully replicate many of the features of AD, including initial intracellular accumulation of Abeta, accompanying cognitive impairment and subsequent appearance of plaques and tau pathology. This model also exhibits neuronal death – a feature present in AD, but absent from many other mouse model systems.

Despite the dominance of murine models of AD, it is clear that the power and speed of the techniques that can be applied to invertebrate systems have provided an impetus to perform *C. elegans* and *Drosophila* studies. Link and colleagues have created a *C. elegans* model of AD based on the secretion of Abeta from cells as a transgenic peptide attached to a secretion signal peptide (70). While Abeta accumulates within the muscle cells of the worm in this model, rather than reaching the extracellular compartment, it remains very useful as it develops a clear phenotype characterized by progressive paralysis. This has been successfully exploited by Dillin and colleagues to dissect the complementary contributions of the heat shock and insulin growth factor-1 like signaling pathways to protection against toxicity and to observe how this balance changes with aging (71).

In many ways the use of worm and fly models is complementary; however *Drosophila* models of AD have some advantages, in particular the fly has a complex brain and hence the complexities of the phenotypes that may be assessed are correspondingly greater (Figure 5). For example the Abeta-expressing fly models allows disease-relevant phenotypes such as learning, memory (72), habituation (73, 74) and locomotor function to be explored in the context of progressive histological disruption (75,

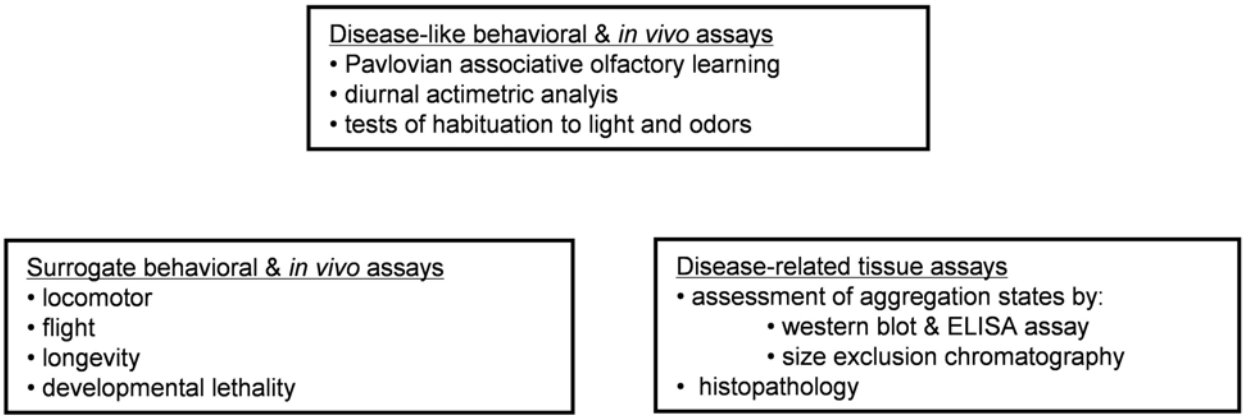

Figure 5. *Drosophila* model of AD. Overview of phenotypes and assays that may be assessed and applied in *Drosophila* models of AD.

76). It is likely that the quantitative analysis of complex behavioral phenotypes such as these will provide markers of pathological progression in the fly that resemble the clinical features of patients with AD. Furthermore, the short lifespan of the fly allows us to look at phenotype progression within days rather than months or years (life spans typical of rodent models) and this, combined with the potential sensitivity of behavioral phenotypes, will allow the rapid generation of data. In particular we find reductions in longevity and climbing ability to be strong indicators of Abeta toxicity. It is of particular interest that functional phenotypes, such as locomotor impairment, are seen early in the life span of the flies, when only intracellular accumulation of Abeta is apparent, and in the absence of mature amyloid (75). Interestingly, biochemical tests on the brains of AD flies demonstrates the presence of SDS-resistant oligomers of Abeta, mirroring similar findings from examination of the human cerebral cortex and CSF (77, 78) and also from studies of the conditioned medium of Chinese hamster ovary cells that have been genetically modified to produce human APP variants (79, 80).

The powerful genetic tools that are available for the fly also allow us to dissect the biological pathways that mediate the toxic response of neurons to Abeta aggregates. By randomly manipulating the expression level of genes in the fly genome we can assess the effects of these genes on the sensitivity of the fly to Abeta toxicity. Cao and colleagues have reported a genetic screen in which a wide range of genes are implicated in the pathogenesis of the AD-related phenotypes, including those associated with secretion, cholesterol homeostasis, and regulation of chromatin structure and function (81). We have also performed genetic screens that have revealed novel modifiers of Abeta toxicity, allowing us to dissect pathways of toxicity in molecular detail.

We can also combine biophysical studies with *Drosophila* modeling to assess the relative pathogenic contributions of the intrinsic aggregation properties of the Abeta peptide and the cellular factors present in the *in vivo* environment (82). Specifically, by performing mutagenic analysis of Abeta behavior *in vitro* and *in vivo*, guided by *in silico* predictions of Aβ aggregation propensity, it is possible to link the generation of particular aggregates with *in vivo* toxicity. Indeed we have found a strong correlation between the propensity of the $Abeta_{42}$ peptide to form protofibrils and neuronal dysfunction in a *Drosophila* model of AD (28). This observation supports the notion that it is the early-formed Abeta aggregates that have potent neurotoxic activity and that these species may be the key effectors of neurotoxicity in AD (83). In fact, there is evidence that the cognitive features of early AD, as well as synaptic loss, correlate better with the amount of soluble Abeta aggregates than with the appearance of insoluble plaques in the brains of AD patients and in mouse models (84, 85).

4. LYSOZYME AND SYSTEMIC AMYLOIDOSIS

In the 1960's, lysozyme was the first enzyme to have its X-ray crystallographic structure determined (86). Since then lysozyme has been extensively studied, using a wide variety of biophysical techniques, with the aim of understanding many features of its behavior including the principles that govern both normal protein folding and pathological misfolding and aggregation (for a more detailed review see (87) and references therein). In the early 1990s, it was found that mutations in the human lysozyme gene cause hereditary non-neuropathic systemic amyloidosis (88). Since the first reported case, four single amino acid mutations (I56T, F57I, W64R, D67H) and two double mutations (F57I/T70N, T70N/W112R) have been linked with this rare autosomal-dominant disease (Figure 6) (89-91). In common with Alzheimer's disease, all these cases of familial disease appear to be characterized by amyloid deposits but, in contrast to AD, the pathogenicity of systemic disease appears to be primarily mediated by the disruption of organ function caused by the bulk accumulation of amyloid fibrils (7, 92). Detailed investigation of patients carrying the D67H lysozyme variant has demonstrated that the deposits are composed solely of the full-length, variant protein (92). Circulating levels of lysozyme have been measured in the plasma and urine of a small number of patients and found to be within

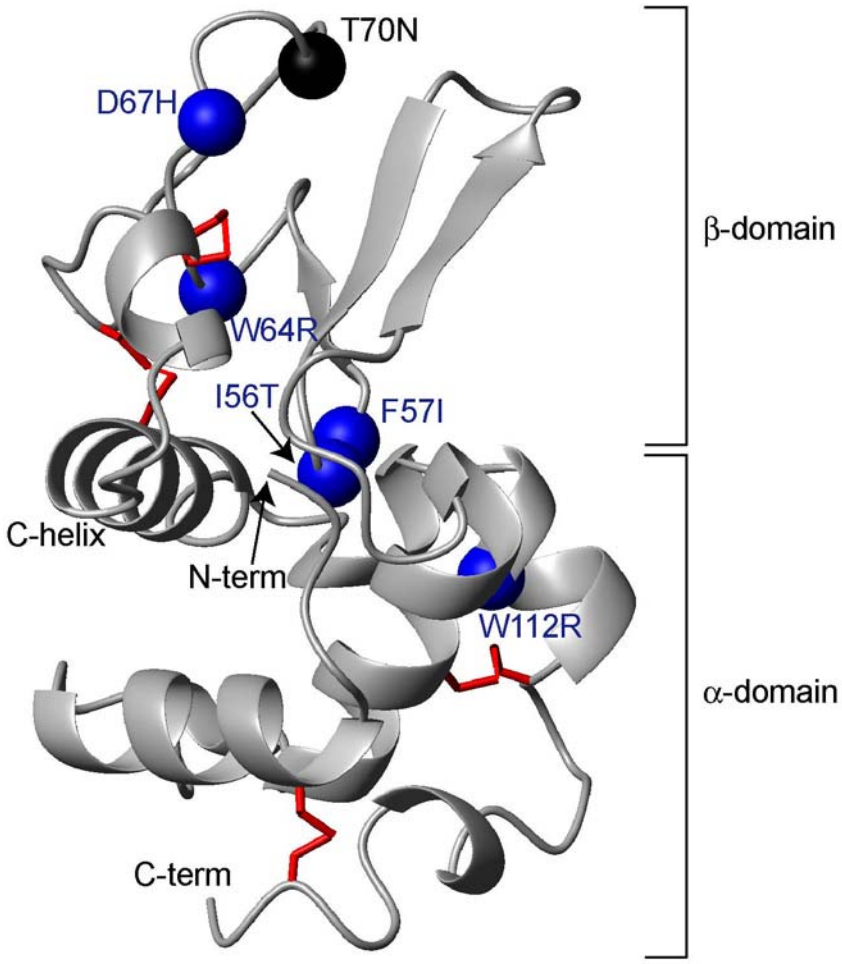

Figure 6. Location of naturally occurring mutations in human lysozyme. A ribbon diagram of the structure of wild-type human lysozyme showing the location of the known natural mutations. The mutations giving rise to amyloidogenic variants are shown in blue and the non-amyloidogenic variants shown in black. W112R has only been detected in conjunction with T70N and not as a single point mutation. The four disulfide bonds are shown in red.

the normal range but, interestingly, patients possessing the W64R mutation have been reported to have only wild-type (WT) lysozyme in their plasma and urine and no detectable levels of the W64R protein (based on analysis by mass spectrometry) (89).

4.1. *In vitro* characterization of lysozyme fibril formation

Variants of human lysozyme (I56T and D67H) have been shown to convert *in vitro* from normally soluble and functional proteins into intractable fibrillar aggregates (92, 93). These fibrillar aggregates resemble those found *in vivo* and have been characterized by a wide variety of techniques including light-scattering, thioflavin T fluorescence spectroscopy, transmission electron microscopy (TEM), X-ray and electron fibre diffraction and Congo-Red staining (94, 95). The kinetics of *in vitro* aggregation show a clear lag phase, followed by a rapid exponential growth phase. It has been shown that the lag phase is however abolished, and the overall rates of fibril growth for I56T, D67H and WT lysozyme are greatly accelerated, by seeding with preformed fibrils (93, 96). These results are consistent with a general nucleation-dependent growth process typical of amyloid formation observed in other systems (37).

From detailed structural studies of the I56T and D67H variants, and to a lesser extent the F57I and W64R variants, it is believed that a decrease in native stability and global structural co-operativity (i.e. with respect to the co-operative two-state transition between the folded and unfolded states of WT lysozyme), leads to the population of a transient, partially unfolded species, and eventually to the formation of fibrils (97-99). This transient intermediate can be monitored by measuring the rates of solvent exchange of labile hydrogens in the protein by mass spectrometry under conditions where the exchange rates are fast compared to the local co-operative unfolding/folding process that generates the aggregation prone transient intermediate (i.e. EX1 conditions (100)). Under these conditions all labile

hydrogens that are exposed will exchange each time a fluctuation occurs. Such behavior is usually indicative of complete global unfolding of the protein structure from the native state; however, a fluctuation resulting in a co-operative unfolding of a region of the protein molecule would result in a well-resolved multimodal distribution of masses (97). Under physiologically relevant conditions of pH and temperature, a bimodal distribution is observed for the I56T and D67H variants; in contrast, WT lysozyme and the naturally occurring, non-amyloidogenic variant T70N do not display such behavior that is indicative of a well-defined transient intermediate (97, 98, 101). This intermediate is remarkably similar for both the I56T and D67H variants and consists of an unfolded beta-domain and C-helix while the rest of the alpha-domain remains folded. Interestingly, it bears a close resemblance to the dominant intermediate detected during non-oxidative folding (97, 102); moreover, this intermediate species is not unique to amyloidogenic variants, and in fact can be populated under non-physiologically relevant conditions (i.e. at higher temperature) in WT lysozyme and the T70N variant (101). Limited proteolytic digestion of fibrils formed at low pH with the homologous hen egg white lysozyme (HEWL) has revealed that regions corresponding to the beta-domain and the C-helix are resistant to enzymatic digestion (103). These fragments rapidly formed amyloid fibrils after purification, suggesting that the region corresponds to a high intrinsic propensity to aggregate and are located in the fibrillar core. These characteristics strongly suggest that *in vivo* aggregation is closely coupled to the ability to readily form the intermediate species under physiological condition.

Based on the structural data, we can rationalize that, for the amyloidogenic variants, the mutations result in the destabilization of the interface between the alpha- and beta-domains, allowing a partial unfolding of the beta-domain and the C-helix and thereby exposing a substantial region of the protein that can, in some conditions, result in intermolecular interactions being favored over the intramolecular interactions characteristic of the native structure (87). In such circumstances, the process of aggregation can be initiated which ultimately leads to the formation of amyloid fibrils.

The ability to follow lysozyme fibril formation *in vitro* provides an excellent opportunity to investigate potential strategies to inhibit aggregation. Our first approach in this direction builds on our understanding that decreased native stability, along with lower co-operativity, results in enhanced amyloidogenicity and has involved the use of a camelid antibody fragment (cAb-HuL-6, raised against WT human lysozyme) that binds to and stabilizes the native protein (96, 98). The binding of cAb-HuL-6 to the native state of D67H or I56T variant lysozyme abolished the formation of the transient intermediate and resulted in the suppression of fibril formation. Structural studies of this complex revealed that the epitope on the lysozyme molecule does not include the sites of mutations or even the region prone to local unfolding (beta-domain). The antibody therefore appears to act by restoring global co-operativity to the native structure and thereby preventing destabilization of the interfacial region through long-range conformational effects. This study demonstrates the crucial nature of maintaining the global co-operative nature within globular proteins (e.g. by preventing the formation of unfolding intermediates) in order to resist aggregation; in this case of lysozyme this study therefore pinpoints the transient intermediate as a key species along the fibril forming pathway (Figure 7) (87).

4.2. Understanding lysozyme aggregation *in vivo*

In biological systems, the evolutionary design of the amino acid sequence of proteins along with other protective mechanisms such as quality control and degradation processes and the presence of molecular chaperones, have evolved to avoid aggregation under normal circumstances (104). The large majority of molecular chaperones are located in the intracellular environment; however, recently three extracellular chaperones, clusterin, haptoglobin (Hp) and alpha$_2$-macroglobulin (alpha$_2$M) have been identified and found to inhibit protein aggregation *in vitro* (105-107). Due to the extracellular nature of the lysozyme fibrils found *in vivo*, lysozyme represented a relevant target for investigating whether the extracellular chaperones could affect fibril formation.

All three proteins are secreted glycoproteins found circulating in blood and other bodily fluids of humans and other mammals. Clusterin, the most well characterized of the three chaperones, is translated as a 449 amino acid residue single polypeptide chain, which has a 22 residue secretory signal that is proteolytically cleaved after biosynthesis (108, 109). Post-translational modifications include N-linked glycosylation and proteolytic cleavage into an alpha- and beta-peptide that are concomitantly linked by five disulfide bonds to form a heterodimer. The precise physiological role of clusterin is unknown, although a diversity of functions has been suggested including its ability to act in a fashion similar to small heat-shock proteins *in vitro* (110, 111). Reports of *in vitro* inhibition of fibril formation by clusterin has been published for a number of peptides and proteins linked with aggregation diseases including the Abeta peptide, apo C-II, and the prion peptide 106-126 (105, 112, 113).

More recently, the chaperone-like ability of Hp and alpha$_2$M has been reported (106, 107). Alpha$_2$M is an abundant human blood glycoprotein, comprised of ~10% carbohydrate by mass. It is best known for its ability to inhibit a broad range of proteases, but has also been found to interact with a number of proteins and peptides involved with deposition diseases (114); indeed it has recently been shown that alpha$_2$M can inhibit fibril formation of Abeta peptide as well as aggregation of a variety of other proteins (107, 115). Hp is produced mainly in the liver and is found in most bodily fluids of humans and other mammals. Like clusterin, levels of Hp in human plasma are increased during various physiological stresses (e.g. inflammation) (108, 116). Hp has been found to protect many different proteins from stress-induced amorphous precipitation and has been found to be associated with Abeta deposits *in vivo* (106, 117).

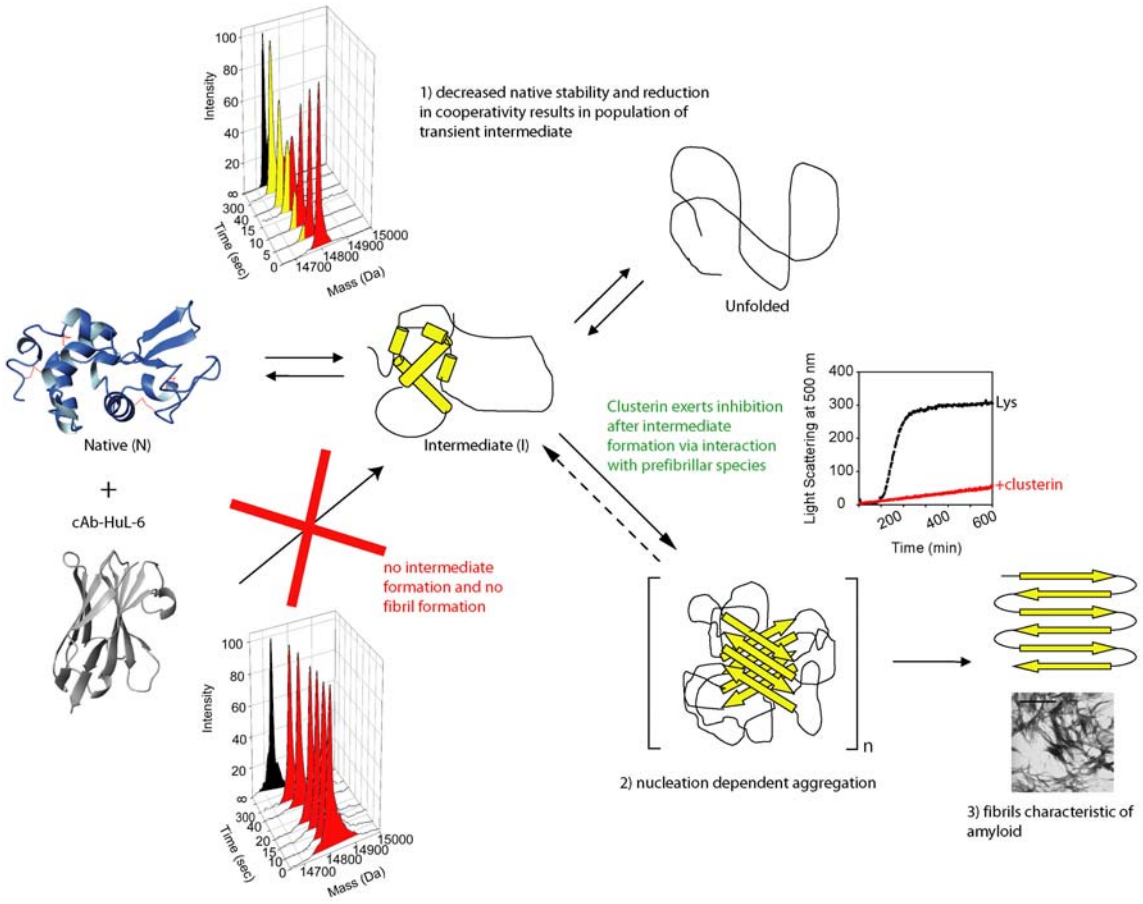

Figure 7. Schematic diagram of lysozyme fibril formation and methods of inhibition. A proposed model for lysozyme fibril formation is shown. A decrease in monomeric native stability coupled to a reduction in co-operativity results in the population of a transient intermediate which can be monitored by mass spectrometry. This intermediate leads to the formation of oligomers and finally to mature fibrils. A single chain antibody fragment has been shown to bind to the native state of lysozyme and inhibit the formation of the transient intermediate, thereby reducing fibril formation. The extracellular chaperone clusterin exerts its inhibitory properties via interactions with prefibrillar species present after the formation of the transient intermediate.

Our initial studies looked at the influence of substoichiometric amounts of clusterin on *in vitro* fibril formation by the amyloidogenic variant, I56T, as well as a non-amyloidogenic variant, I59T, which possesses many characteristics of the amyloidogenic variants. It was found that clusterin could potently inhibit lysozyme fibril formation even at molar ratios of clusterin-to-lysozyme of 1:80 (Figure 8) (99). It was further observed that clusterin did not detectably interact with the native or fibrillar states of lysozyme, nor did it influence the formation of the monomeric transient intermediate as observed by mass spectrometry. Moreover, addition of substoichiometric amounts of clusterin at the beginning of the aggregation reaction resulted in complete inhibition whereas addition at a later stage (mid-elongation) resulted in a much less effective inhibition. From this study, it appears that the clusterin interacts with oligomeric species that are present at low concentrations during the lag phase of the aggregation reaction and in this way it inhibits fibril formation by lysozyme *in vitro*, a result that strongly support the role of the transiently populated aggregation intermediates discussed above. These findings have been supported by investigations with other fibril forming proteins (113).

Recently we have also examined the effects of Hp and alpha$_2$M on lysozyme fibril formation *in vitro* and have found that they too can inhibit the aggregation process (118). The ability of these extracellular chaperones to potently inhibit *in vitro* fibril formation by human lysozyme variants, as well as other disease related peptides and proteins, is of great significance as this suggests that in a biological system they could play a key role in restricting the potentially pathogenic effects of the aggregation of proteins that are secreted into the extracellular environment.

Until recently, attention has been focused on understanding the mechanism of *in vitro* fibril formation by the amyloidogenic variants of human lysozyme at a molecular level. However, a full appreciation of the origins of pathogenesis requires more complex model systems and

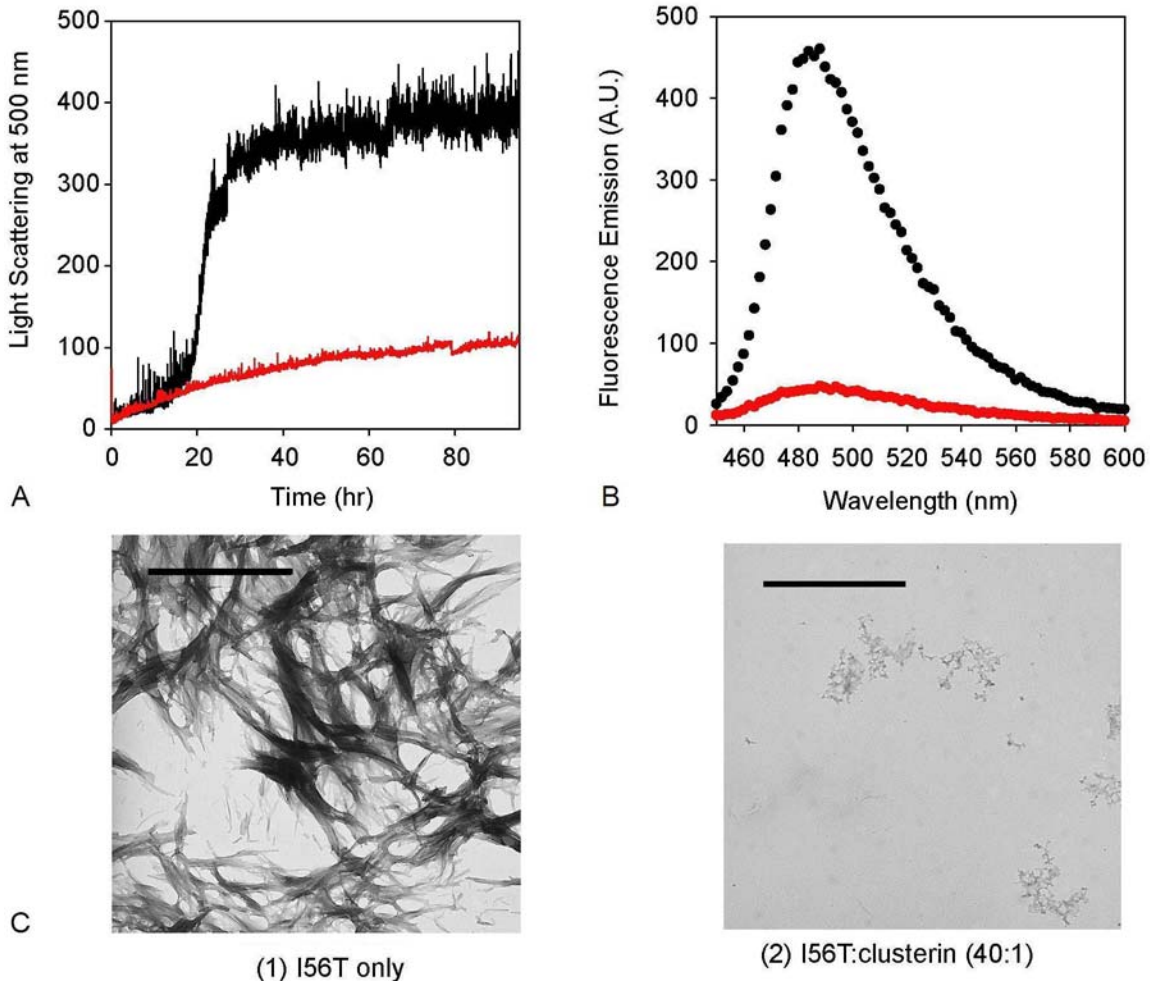

Figure 8. Inhibition of lysozyme fibril formation by the extracellular chaperone clusterin. A) Light scattering resulting from I56T lysozyme aggregation in the absence (black) and presence of clusterin (red) at a 1:40 clusterin-to-lysozyme molar ratio. B) Thioflavin T fluorescence measured at the end of the growth phase observed in the light-scattering assay for I56T lysozyme alone (black) and I56T lysozyme in the presence of clusterin (red). C) TEM analysis of samples taken at the end of the growth phase observed in the light scattering assay. The scale bar represents 1 micrometer.

to achieve this goal we have examined the behavior of amyloidogenic lysozyme variants in yeast (*P. pastoris*) and *Drosophila* ((119), manuscript in preparation). Our initial experiments have examined the levels of secretion of WT and disease-associated variants of lysozyme. Because the toxicity of lysozyme amyloidosis results from the effect of large, extracellular deposits containing full-length, protein, one might assume that the variant protein is capable of bypassing the normal quality control mechanisms, resulting in high level secretion of these proteins that subsequently deposits systemically. We have found, however, when expressing lysozyme in *Pichia pastoris,* the level of secreted protein is lower for destabilized variants of lysozyme (Figure 9) (119). In line with these data we have also found, by using *Drosophila* as a model organism of lysozyme amyloidosis, that the level of soluble lysozyme is much lower for the disease associated variants than for the WT protein (unpublished data). By using a *Drosophila* model it is possible to undertake tissue selective studies in an age dependent manner to examine how the onset and severity of the disease is affected by the complex interplay of various factors including reduced stability and increased aggregation propensity of the protein and the effectiveness of the quality control system in the cell.

The relationship between stability and secretion level observed in these *in vivo* models suggests that, in a normally functioning organism, a delicate balance exists between the efficiency of the secretory processes and the nature of the destabilization of the protein, caused by a mutation, that is crucial in determining whether or not specific variants are able to accumulate in the tissue. This suggests that other biological processes may contribute to the development and severity of this disease.

5. SERPINS AND FAMILIAL ENCEPHALOPATHY WITH NEUROSERPIN INCLUSION BODIES

The autosomal dominant dementia, familial

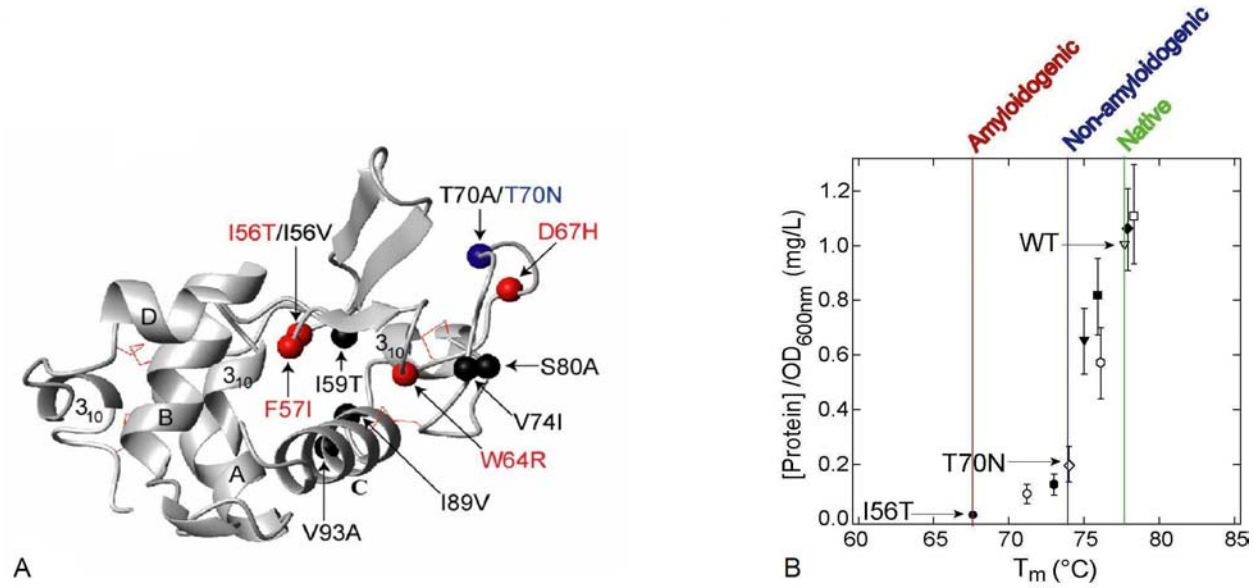

Figure 9. Relationship between the native stability of lysozyme and its secretion in *P. pastoris.* A) Location of point mutations of the variants used to analyze protein secretion in *P. pastoris.* Amyloidogenic variants are shown in red, the non-amyloidogenic variant is shown in blue and the non-natural variants are shown in black. B) Relationship of native stability as measured by thermal denaturation (T_m) and levels of lysozyme secreted by *P. pastoris*. The variants include I56T (filled circle), I59T (empty circle), T70A (filled hexagon), T70N (empty diamond), I56V (filled triangle), I89V (filled square), V93A (empty hexagon), WT (empty triangle), S80A (empty diamond) and V74I (empty square). Reproduced with permission from reference (119).

encephalopathy with neuroserpin inclusion bodies (FENIB), is caused by mutations in neuroserpin, a neuronal serine protease inhibitor (serpin) (120). FENIB results from the accumulation of neuroserpin polymers and is remarkable because of the strong genotype-phenotype correlation that is observed in various kindreds, whereby the mutations that most strongly promote neuroserpin aggregation have the most profound clinical sequelae (121). The handling and toxicity of neuroserpin polymers has been investigated using a range of methods both *in vitro* and *in vivo* using both cell and animal models of FENIB.

5.1. Serpins and the serpinopathies

The serpins are an abundant and widespread group of proteins characterized by greater than 30% amino acid sequence homology with the archetype alpha$_1$-antitrypsin. Found in all major forms of life from viruses to humans, they fulfill a large number of diverse roles by utilizing a unique mechanism of protease inhibitory action (122, 123). The structure of the serpins is centered on three beta-sheets (A-C) and a flexible reactive centre loop (RCL) which is exposed at the top of the molecule (Figure 10A) (124). This reactive centre loop and the largest beta sheet, beta-sheet A, interact to snare and inactivate the target protease in a unique and dramatic mousetrap action (125). The reactive loop acts as a pseudo-substrate to which the target protease docks. The protease then cleaves the reactive centre loop which triggers a large molecular transformation in the serpin. The reactive centre loop is incorporated as an extra strand in beta-sheet A and swings the protease from one pole of the serpin to the other, irreversibly inactivating it and targeting it for degradation.

The unique mechanism of inhibition exhibited by the serpins is highly effective but the metastability of their native structures and the large molecular transformations that occur during protease inhibition leaves them vulnerable to mutations that destabilize the molecule and result in the formation of polymers. The accumulation of variant serpin polymers is associated with a group of diseases termed the serpinopathies (126). The serpinopathies exhibit two pathological mechanisms, firstly, the accumulation of serpin polymers within the organ of synthesis results in local damage and, secondly, there may be lack of circulating serpin (126). The clinical features of different serpinopathies depend on both the organ of serpin synthesis and its target tissue. For example, alpha$_1$-antitrypsin is synthesized in the liver and inhibits neutrophil elastase in the lung (127). Mutations that cause alpha$_1$-antitrypsin to polymerize result in the retention of the protein within the liver and hence, cirrhosis and emphysema of the lungs develop due to a lack of circulating alpha$_1$-antitrypsin and unchecked protease activity (128).

Two mechanisms for the formation of serpin polymers have been proposed, and both models involve an expansion of beta-sheet A and a domain swap between serpin molecules into beta-sheet A. The first model for the formation of serpin polymers was proposed by Lomas and colleagues in 1992, and recently Huntington and co-workers have solved a new crystal structure suggesting that two beta-strands are involved in the domain swap (129-130). Vitally, both models describe polymers which are composed of correctly folded monomers in contrast to the protein aggregates of other conformational diseases in

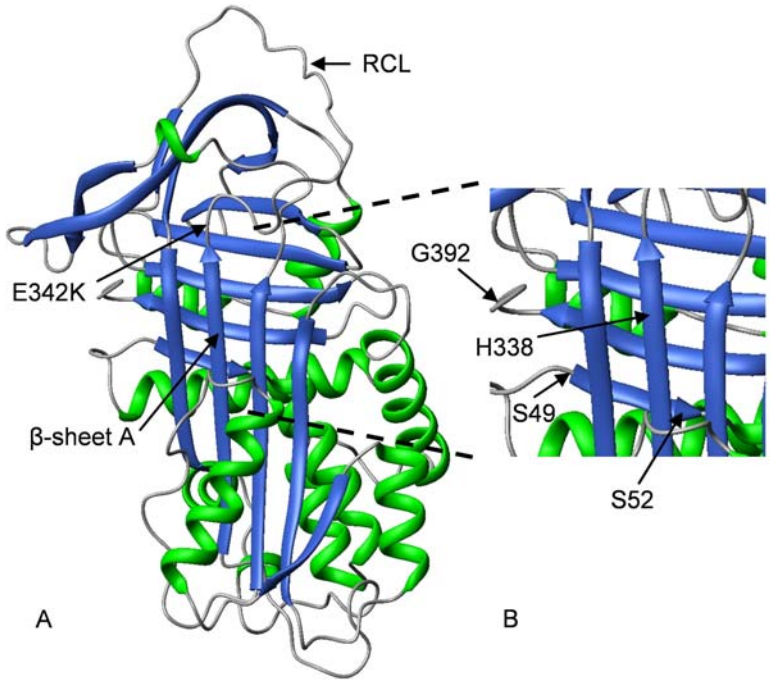

Figure 10. The structure of serpins. A) The structure of alpha$_1$-antitrypsin is shown which exemplifies the structure of the serpins (PDB code: 2QUG). Marked are the reactive centre loop (RCL), beta-sheet A and the mutation E342K. B) The shutter region is magnified and residues that are mutated in FENIB are highlighted.

which the native structures of the individual monomers are partially or completely lost when they are assembled into amyloid fibrils (2).

Under certain circumstances, the RCL of a serpin molecule is incorporated into the centre of its main beta-sheet A, without cleavage of the loop. This results in a "latent" conformation of the protein which is more stable than the active conformation in which the RCL is solvent exposed at the top of the molecule (131-134). Mutations that destabilize this serpin architecture make the protein highly susceptible to intermolecular interactions, in which the RCL of one serpin monomer is incorporated into beta-sheet A of another serpin monomer resulting in chains of loop-beta-sheet polymers.

The molecular basis of the polymerization for the Z variant of alpha$_1$-antitrypsin (E342K) has been studied in detail (128, 129, 135-138). The E342K mutation is located at the C-terminal end of beta-strand 5 of beta-sheet A and at the base of the RCL (Figure 10A). This mutation causes a conformational transition resulting in the formation of an unstable intermediate (denoted M*) where the RCL is only partially inserted into beta-sheet A. This event causes an expansion of beta-sheet A, thereby favoring the incorporation of the RCL of another molecule resulting in the formation of a loop-sheet dimer which extends to form longer chains of loop-sheet polymers (129, 136, 137, 139-142).

5.2. Neuroserpin and FENIB

Neuroserpin is expressed in both developing and adult brains in areas of synaptic plasticity and migrating neurons where it inhibits its target protease tissue-type plasminogen activator (tPA) (143-147). Through its interaction with tPA, and potentially through roles independent of tPA, neuroserpin is involved in modulating synaptogenesis and syntaptic plasticity, in memory, emotion, behavior and in protection from ischemic injury (148-152). However, in FENIB, mutations in neuroserpin (Figure 10B) cause it to gain a toxic function, the propensity to form polymers.

Mutations in neuroserpin cause the retention of neuroserpin polymers in neurons resulting in the autosomal dominant dementia FENIB (120). Immunohistochemical staining of brain sections reveals neuroserpin polymers in PAS-positive inclusions (known as Collins bodies) that accumulate in the deeper layers of the cortex and the substantia nigra (120). By using molecular modeling, the five mutations in neuroserpin, that have been linked with FENIB, are predicted to disrupt the structure of neuroserpin and, to differing extents, favor the opening of beta-sheet A and the consequent formation of loop-sheet polymers, in a similar fashion to the polymerization described for Z alpha$_1$-antitrypsin (153). Strikingly, the predicted molecular instability for each neuroserpin variant correlates well with its *in vitro* polymerization rate, the number of inclusions formed in the brain, the clinical severity of the disease and a decreasing age of onset (121, 153, 154). Specifically, the S49P Syracuse mutation introduces the cyclic, uncharged residue proline beneath beta-sheet A, whilst the S52R Portland mutation introduces the more extended, charged residue arginine in this same region. Individuals carrying the S49P mutation develop dementia and tremors between the ages of 45 and 60 whilst the S52R mutation causes

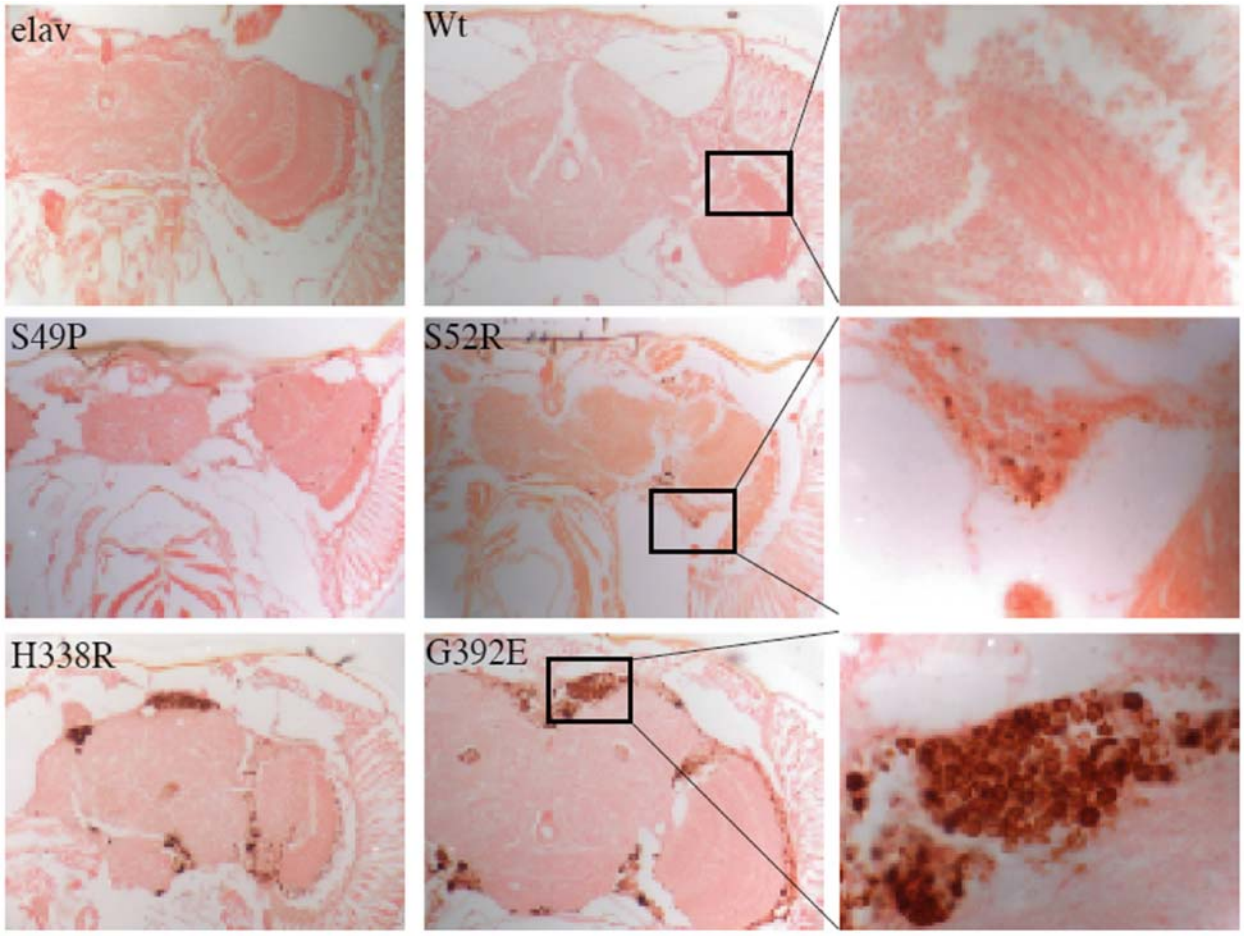

Figure 11. Variant neuroserpin accumulates in neurons. Intracellular accumulation of neuroserpin variants (brown staining) was located within cortical neuronal cell bodies, adjacent to the mushroom bodies and lobula. Left and middle panels were taken with a 20X objective, enlarged details in right panels were obtained with a 100X oil immersion objective. Sections were stained with a mouse monoclonal antibody that detects total neuroserpin. Reproduced with permission from reference (157).

dementia, myoclonus and status epilepticus with onset during the third and fourth decades of life (120). These mutations affect residues beneath beta-sheet A, but a more severe form of the disease is caused by mutations affecting residues within beta-sheet A itself. His338 of strand 5 forms essential hydrogen bonds that stabilize strands 3 and 5 with beta-sheet A in a closed confirmation. The mutation H338R obliterates these bonds, resulting in an opening of beta-sheet A and a more severe disease phenotype, with progressive myoclonus epilepsy and dysarthia at 15 years of age (121). This region is disrupted to yet a greater extent by mutation of residue 392. Normally, the glycine residue at this position allows compact positioning of the strands of beta-sheet A; however, the introduction of glutamic acid with the mutation G392E greatly favors the opening of beta-sheet A, resulting in progressive myoclonus epilepsy, dysarthia and chorea at 13 years of age (121). Finally, the G392R mutation introduces the larger arginine residue at the same position and causes the most severe form of FENIB with the earliest onset, at 8 years of age (154). It is possible that the epilepsy associated with FENIB is caused by a lack of circulating neuroserpin, although neuroserpin-knock out mice show no seizure phenotype (151, 153); however, dementia and neurodegeneration is a result of the accumulation of unique polymers of correctly folded neuroserpin.

5.3. Characterization of neuroserpin polymerization *in vitro* and in cell culture

The quantitative measurement of the *in vitro* aggregation rates of neuroserpin variants has allowed us to clearly link the molecular behavior of this specific protein to the clinical consequences of the mutations. Accordingly, the S49P and S52R neuroserpins form polymers *in vitro* much more rapidly than does the wild type (WT) protein, with the clinically most severe mutation, S52R, exhibiting the fastest rate of polymerization (153, 155). The genotype-phenotype correlation has been further investigated in cell cultures by expressing neuroserpin variants transiently in COS-7 cells and in stably transfected PC-12 cells. Here we see increasing intracellular retention of neuroserpins as we express increasingly polymerogenic variants (156, 157).Expression in PC-12 cells has shown that variant neuroserpins are retained within the endoplasmic reticulum

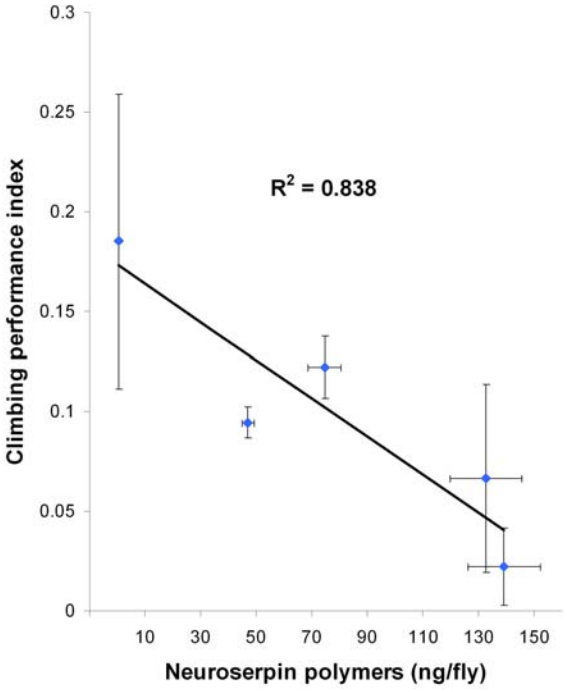

Figure 12. Accumulation of neuroserpin polymers correlates with a loss of climbing ability in flies at 35 days of age. Each point represents flies of a single genotype. Three aliquots of 5 flies were used for ELISA and three aliquots of 15 flies were used for climbing assays. Error bars show standard deviations. All flies were able to climb well on the day of eclosion, and the performance index is 0.76 – 0.86. Reproduced with permission from reference (157).

and that polymers co-localize with the ER marker calreticulin, whereas WT neuroserpin is not retained in the ER but instead co-localizes with GM130, in the Golgi, and then chromogranin A, in the dense-core secretory vesicles, demonstrating successful trafficking through the regulated secretion pathway. This conclusion is confirmed by the observation that G392E neuroserpin from PC-12 cell lysates is sensitive to endoglycosidase H treatment, as revealed by a reduction in its apparent molecular mass after such treatment. In contrast, WT neuroserpin is insensitive to this treatment and the S52R neuroserpin exhibits an intermediate behavior with a fraction of the protein being sensitive to the endoglycosidase treatment. Here we see a subcellular genotype-phenotype correlation, with more severe mutations resulting in earlier retention in the secretory pathway.

5.4. Animal models of FENIB

A number of animal models of FENIB have also provided important insights into the disease. The first, a rat model using over-expression of wild type megsin, a kidney serpin, has demonstrated the existence of intraneuronal inclusions, up-regulation of ER stress proteins, activation of caspases-12 and -3, loss of neurons and a loss of motor coordination (158). Arguably, the expression of human neuroserpin is required for a faithful model of FENIB and consequently mice have been generated that express WT, S49P and S52R neuroserpins under the Thy-1 promoter (159). These mice accumulate neuroserpins within intraneuronal inclusions and develop a range of neurological symptoms including ataxia and coordination deficits. Mice expressing variant neuroserpins lose their ability to grasp a horizontal wire and to climb a grid of negative geotaxis, and are found to be more passive during handling. The observed accumulation and clinical symptoms correlate with the genotypes, whereby mice expressing S52R generally accumulate more neuroserpin, and exhibit more severe symptoms, including tremor and seizure-like episodes, earlier in their lives than the mice expressing S49P. Interestingly, it has been found that inclusions are present in the brain prior to clinical symptoms.

We have developed a *Drosophila* model of FENIB by expressing WT and variant neuroserpins in the central nervous system of the flies using the pan-neuronal driver *elav-Gal4* (157). Using a sandwich ELISA assay the accumulation of neuroserpins is seen to recapitulate the genotype-to-phenotype correlation observed in human disease. Expression of increasingly polymerogenic variants of neuroserpin results in an increasing accumulation of neuroserpins in the brains of the flies. Immunohistochemical analysis of neuroserpin accumulation in the flies' brains reveals strong staining within cortical neuronal cell bodies, adjacent to the mushroom bodies and lobula, again with the most severe variants showing the highest level of accumulation (Figure 11). In addition, when a monoclonal antibody specific for polymers of neuroserpin is used in the ELISA assay, it can be seen that polymers are generated in flies expressing variant neuroserpins, but not in flies expressing WT neuroserpin. The accumulation of these polymers is detrimental to the flies and they exhibit an age-dependent loss of locomotor ability that correlates with the accumulation of neuroserpin as measured by ELISA (Figure 12). This model provides an opportunity to perform a genetic screen for modifiers of the accumulation of variant neuroserpins and may help us to understand the pathways and processes that handle these unique protein polymers.

6. PERSPECTIVE

In the crowded macromolecular environment of the cytoplasm, the ability of proteins to fold and function without forming aberrant intra- or intermolecular interactions is of crucial importance. There is increasing evidence that the need to maintain protein solubility provides a major evolutionary pressure on protein-coding DNA (160, 161). One common consequence of protein aggregation is the formation of amyloid deposits and these can cause disease either by the cytotoxic action of soluble aggregates, as in AD, or by the effect of the deposition in vital organs of large quantities of protein, as in the systemic amyloidoses. The serpinopathies represent an alternative pathogenic mechanism, in which the intracellular accumulation of near-natively folded proteins can cause disease. The methods and models that we have described

for these representative proteins are applicable to a wider range of protein misfolding problems and provide vital opportunities for exploring therapeutic strategies, in addition to enhancing our knowledge of the delicate balance between the normal and pathological interactions behind protein misfolding.

7. ACKNOWLEDGMENTS

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