

A Central Role for Protein Aggregation in Neurodegenerative Disease; Mechanistic and Structural Studies of Human Stefins

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Abstract

Common cellular and molecular mechanisms underlie different neurodegenerative diseases, from Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis to sporadic prion diseases. The molecular mechanisms include aberrant protein folding and aggregation in the form of extracellular plaques or intracellular inclusions. Deeper understanding of the detailed mechanism of protein aggregation and cellular toxicity should lead to rational drug design for this type of disease. Our studies of human stefin B, a model amyloidogenic protein, will be reviewed. The studies range from establishing the mechanism, imaging the fibrillar and prefibrillar species by transmission electron microscopy (TEM) and atomic force microscopy (AFM), to structural studies of the precursor oligomeric states.

Key words: amyloid-fibril, conformational disease, cystatins, domain-swapped dimer, protein folding

Protein aggregation in neurodegenerative disease

Neurodegenerative diseases comprise Alzheimer's disease (AD), Parkinson's disease (PD), dementia with Lewy bodies, fronto-temporal dementia with Parkinsonism, i.e.: Pick's disease, amyotrophic lateral sclerosis (ALS), polyglutamine extension diseases and prion diseases.¹ The hallmarks of all these diseases are intracellular inclusions made largely of aggregates of selected proteins (Table 1), which leads to degeneration in specific regions of the brain.

Strong evidence for protein aggregation being central to neurodegenerative diseases comes from transgenic animal models that reproduce the neuro-

degenerative pathology. For example, mice with the human gene for mutant synuclein or with the human gene for mutant superoxide dismutase (SOD), reproduce the main features of Parkinsonism or amyotrophic lateral sclerosis. More animal models exist for other neurodegenerative diseases.²

However, most cases are sporadic, usually occurring with age, with environmental factors being important. Life style, exposure to toxins (metals, pesticides, organophosphates), infection and fever, all may increase the likelihood of disease. Systems for cell maintenance exist that prevent even further damage. These can counterbalance increased levels of protein aggregates but only to a certain extent. The main two systems are heat shock proteins (with chaperones and cellular anti-oxidants) and the ubiquitin-proteasome system (UPS).

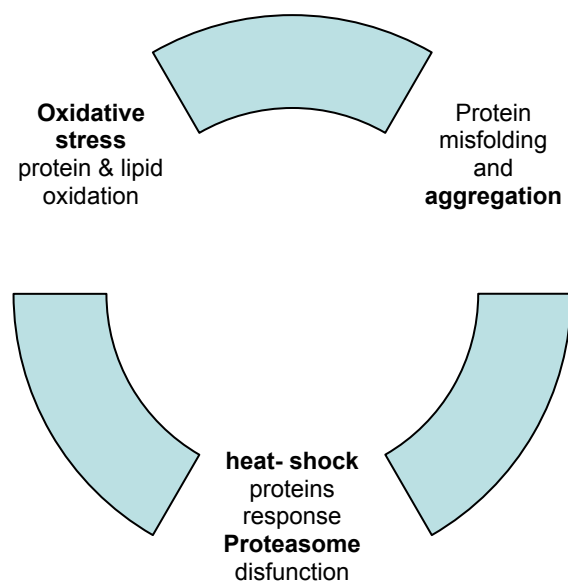
Table 1. Protein fibrillar inclusions in various conformational diseases.

Neurodegenerative disease	Protein component	Cellular inclusion
Alzheimer's disease	tau, A42 β peptide	Neurofibrillary tangles
Parkinson's disease	α -synuclein, crystallines	Neurofilaments/cytoplasm
Lewy bodies dementia	α -synuclein	Lewy bodies/cytoplasm
Pick's disease	tau	Pick bodies/cytoplasm
Progressive supranuclear palsy	tau, heat shock proteins	Neurofibrillary tangles
Amyotrophic lateral sclerosis	Superoxide dismutase (SOD)	Intracellular inclusions
Huntington's disease	Huntingtin, expanded Glu repeats	Intranuclear inclusions
Spinocerebellar ataxias	Expanded Glu repeats of ataxins-1,-3,-7	Intranuclear inclusions
Transmissible spongiform encephalopathies	Prion protein, cathepsin B	Endosome-like organelles

With more extensive protein aggregation, both systems become overwhelmed and the toxic cascade starts.³

Cellular events involved in neurodegeneration range from oxidative stress, increased metal load, reduced level of cellular anti-oxidants, aberrant signalling, membrane permeation and mitochondrion dysfunction to programmed cell death. The order of events is still disputed. It is possible that the cause and effect relations are circular: increased oxidative stress alters the proteins in such a way to render them more amyloidogenic, whereas proteins involved in amyloid-fibril formation might be themselves producing oxidative stress. Oxidative stress further influences glial cell activation (an inflammatory response), mitochondrial dysfunction and programmed cell death (Chart)

Chart. (<http://www.nature.com/focus/neurodegen/>).



Alzheimer's disease as a prototype for neurodegeneration

Much effort and money is devoted to research on Alzheimer's disease (AD) in Europe, and, especially so in the USA. This is not surprising, as AD is the most prevalent dementia in the rich world ageing population. Final aim is to produce a cure or at least to stop disease progress. It is held that revealing the molecular mechanisms of neurodegeneration would lead to a cure.⁴ This is being partially fulfilled but is still far from expectation.

The pathology of AD^{5,6} is closely connected to a few proteins, one of them being the amyloid precursor protein (APP), a transmembrane protein of important, but unknown function. APP is cleaved by several aspartic proteinases: α , β , and γ secretases. Secretases β and

γ cleave the substrate within the membrane in complex with presenilins and produce the famous amyloid- β peptide ($A\beta$) of 40 to 42 aminoacids in length. This peptide, which in smaller amounts might be neuroprotective,⁵ at higher concentrations aggregates and accumulates in the amyloid plaques (also termed senile plaques) in the blood vessels of AD brain. Mutations in APP, secretases or presenilins were found in familial cases of AD, usually leading to a larger amounts of the more fibrillogenic $A\beta$ peptide (1-42).⁵

The »amyloid cascade« hypothesis states that amyloid plaques initiate a cascade of events leading to final pathology of the disease. However, no clear correlation was found between the extent of plaque accumulation and the disease symptoms, in particular for the mild cognitive decline. A more robust correlation is reported between the levels of soluble (oligomeric) $A\beta$ and the severity of cognitive impairment.^{5,6} This led to modification of the "amyloid cascade" hypothesis, stating that most dangerous species for initiation of pathology are proto-fibrils⁷ or even pre-fibrillar species, the so called amyloid derived diffusible ligands (ADDLs).⁸ The diffusible ligands are spherical in shape and range in diameter from 5 to 15 nm, depending on the protein which oligomerizes. Usually, this is equivalent to tetramers up to 32-mers (E. Ž., unpublished observation).

The "channel hypothesis"⁹ states that the prefibrillar oligomers initiate the cascade of events by interaction with membranes – probably making pores – which leads to increase of intracellular Ca-ion and free radical levels, which further leads to changes in cellular components. Oxidatively damaged and over-phosphorylated proteins are more prone to aggregation. In AD, tau, a microtubule binding protein, aggregates in the cell producing the well-known neurofibrillary tangles. Similar inclusions of other proteins are found in sporadic Parkinson's disease, in dementia with Lewy bodies (α -synuclein), in Huntington's disease (huntingtin) or in amyotrophic lateral sclerosis (superoxide dismutase - SOD) (Table 1). That protein aggregation is the main initial trigger has been shown by familial cases, for which mutations of the proteins decrease protein stability and perturb its folding. In most cases, the mutations lead to extended accumulation of pre-fibrillar aggregates (which are more toxic than the mature fibrils themselves).

What is known about the structure of amyloid-fibrils

Amyloid fibrils exhibit certain distinctive features. They are long fibril entities (μm range) with lateral dimensions in the range of 6–13 nm with a distinctive X-ray diffraction fingerprint that results from the cross- β structure (Figure 1).^{10,11}

Amyloid-fibrils share a common molecular skeleton, the protofilament core structure, which is a continuous β -sheet helix.¹² The X-ray diffraction reflections at approximately 4.7 Å on the meridian and 10 Å on the equator are seen in all amyloid fibre diffraction patterns. The structural repeat of 4.7 Å along the fibre axis corresponds to the spacing of β strands and the 10–12 Å spacing corresponds to the face to face separation of the β sheets (Figure 1).^{10,13}

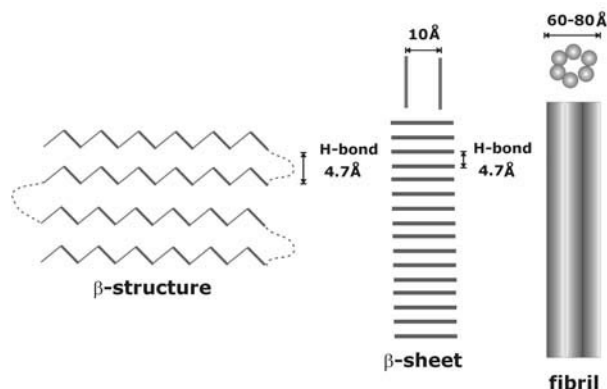


Figure 1. Hierarchy of structure from protein folded into a β -pleated structure to amyloid fibril. The 4.7 Å correspond to the hydrogen bonding distance between β -strands, the 11 Å correspond to the distance between two β -sheets and the 60–80 Å distance corresponds to an average fibril diameter.¹⁰

The β -sheet structure, which builds up the filament, is made of β strands in either parallel or antiparallel alignment.¹⁴ The mature fibril (Figure 1) is built from 4–6 filaments. Most detailed information on the structure of amyloid fibrils has been obtained using solid state NMR (reviewed by Tycko¹⁴). Recent advances in solid-state NMR instrumentation and methodology have permitted determination of backbone and precise conformation of the side chains of Alzheimer's A β -peptide and transthyretin inside the amyloid fibril.

Models for amyloid-fibrillation: looking for general traits

There is now an increased understanding of the pathways involved in protein aggregation and of the molecular mechanisms of cellular toxicity. Both processes are believed to be common to most neurodegenerative diseases.¹⁵

Even proteins not involved in any known amyloidogenic disease have been shown to aggregate and exert toxicity in a very similar way to pathological proteins.^{16,17} That is one reason why we have embarked on studying the fibrillation mechanism of human stefin B, which serves as a very suitable model protein.

Several models for protein aggregation have been described^{18,19} recent literature stressing most the

nucleated conformational conversion (NCC) model²⁰ and the off-pathway folding model.²¹ The nucleated conformational conversion model states that conformational change is the rate-determining step, rather than oligomer growth, but that the change only occurs on the nucleus of preformed oligomers. The off-pathway folding model states that aggregation is the dead end of an irreversible folding pathway.

Among the newer models²² the model of »critical oligomers« (Modler et al.)²³ and the »dipole assembly model« of Xu et al.²⁴ are important. The first proposes that the first step in amyloid fibrillation reaction is formation of a critical oligomer, built from globular entities, which is followed by coalescence of the critical oligomers into protofibrils (necklace-like structures). The second model²⁴ states that the first step, driven by hydrophobic interactions, is formation of nucleation units. The nucleation units have an intrinsic dipole moment and therefore aggregate linearly forming amyloid fibres. Another model of »domain-swapping« as the basis for amyloid fibril formation is supported by structural evidence on domain-swapped oligomers found for quite a number of amyloidogenic proteins, among them cystatins.^{25,26} A domain swapped dimer is defined as two monomer-like entities composed of parts from different molecules.

What is needed is a rigorous test of the models. To choose between the models protein concentration and temperature effects are of major importance.²⁰ Capturing temporal evolution of morphology by imaging techniques, such as atomic force microscopy (AFM) or transmission electron microscopy (TEM) is also useful (see, figures 2 and 3) but it has certain limitations. The species, which stick to the grid, are not necessarily present in solution in the same proportions. If fibrils grow *in situ* on the AFM mica, again there is some doubt as to whether the process is affected by the surface.

Anyhow, if the hypothesis of a »generic« mechanism of amyloid fibril formation (proposed by Dobson and co-workers)¹⁵ holds true, one common model may be valid for most proteins.

A case study: stefin B amyloid fibrillation

Human stefin B is a cysteine proteinase inhibitor. It is an intracellular protein, expressed in many types of cell, located in the cytoplasm and the nucleus. It has been found as part of a multiprotein complex specific to the central nervous system,²⁷ with none of the interacting proteins being cysteine proteinases. This would indicate alternative function(s) for this protein. The main pathology for this proteinase inhibitor is a rare monogenic epilepsy, a progressive myoclonus epilepsy of type 1

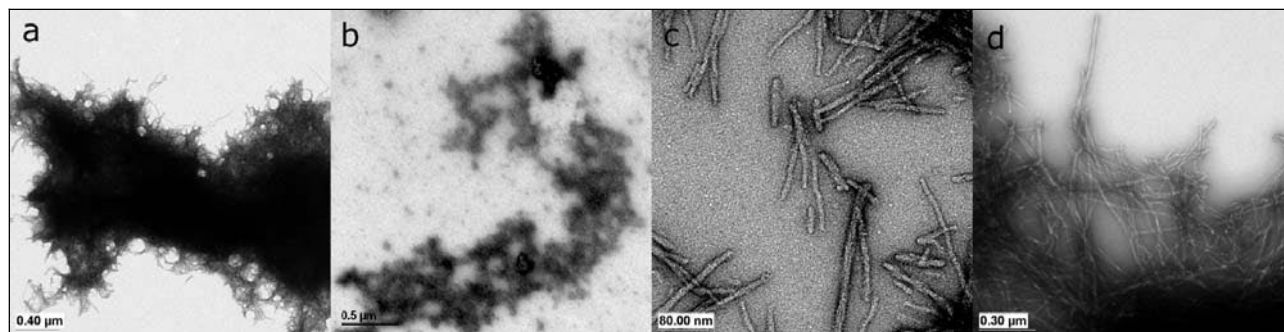


Figure 2. Some typical morphologies on the course of amyloid-fibril formation by human stefin B; **a** amorphous aggregate, **b** granular aggregate, **c** protofibrils, **d** mature fibrils (courtesy of Valentina Zavašnik-Bergant and Kenneth Goldie).

(EPM1), termed Unverricht-Lundborg disease.²⁸ In all clinical cases, due to very different mutations in the gene promoter or exon, lower expression of the protein was found. No amyloid pathology is known for human stefin B (cystatin B) thus far, however, the closely related human cystatin C is a well-known amyloidogenic protein.

The recombinant human stefin B was shown to be very much prone to form amyloid-like fibrils *in vitro*.^{29–31} Its pH dependence of fibrillation was similar to the A β , the pathological peptide of Alzheimer's disease,³² which correlates with the acid-induced intermediates of stefin B shown before.³³

Folding studies of both stefins^{34–36} and their chimeric mutants^{37,38} have been performed. No correlation between the propensity to fibrillate of the chimeras and the stability or folding rates could be shown. Instead, the propensity to fibrillate (Žerovnik et al., in preparation), appears to correlate with the presence of the β -sheet of stefin B.

Stefin B amyloid fibrillation can be induced by lowering the pH below 5 or by adding sulphate at pH 3, which transforms the protein to acid-induced states: the native-like intermediate - I_N and the “molten globule” – MG.³³ The process can be accelerated by pre-denaturing concentrations of the organic solvent TFE^{29,31} or by mutation.³⁹ We have recorded TEM and AFM data in the course of fibrillation and have detected both amorphous and granular aggregates, depending on conditions, and different fibril morphologies. Some of these are shown in Figure 2.

Of interest, we also observed accelerated fibril growth in a 2T magnetic field in cases where we added preformed fibrils as a seed to stefins A and B.⁴⁰ In the magnetic field with the seed added, fibrils grew 3 times faster in the case of stefin A and 50 times faster in the case of stefin B than with seeded reactions occurring outside the field. This is in accordance with hypothesis that a slow nucleation step, which can be accelerated by seeding, is one of the key features of amyloid fibril formation.²²

Comparison of morphological and structural properties of amyloid-like fibrils by stefins B and A

As mentioned, probably all proteins can form amyloid fibrils under appropriate conditions.¹⁵ Therefore, we compared morphological and structural data of amyloid-fibrils of the two homologous proteins stefins A and B. Although topologically similar, stefins A and B fold by different folding pathways and exhibit different propensities for amyloid fibril formation.^{34–36,30,40}

Using atomic force microscopy we demonstrated that both stefins share a common morphology, suggesting structures characteristic of other amyloid fibrils. Stefin A forms two types of fibril that differ in their height (2.8 nm and 5.6 nm), whereas all fibrils of stefin B have the same height (3.4 nm). The length of both fibrils varies from 50 nm up to several microns and all the fibrils show longitudinal periodicity of approx. 26 nm.

Although stefins A and B form fibrils with similar structures (Figure 3), the conditions needed to undergo fibrillation differ. Stefin B fibrils were grown under mild conditions at pH slightly below 5, whereas fibril formation in the case of stefin A can only be induced by reducing pH of a preheated sample dissolved at pH 7, to pH 2.4.^{30,40} Heating stefin A (86 °C for 2 hours) transforms the protein to a domain-swapped dimer, which is separated from the monomer by a high energy barrier.⁴¹

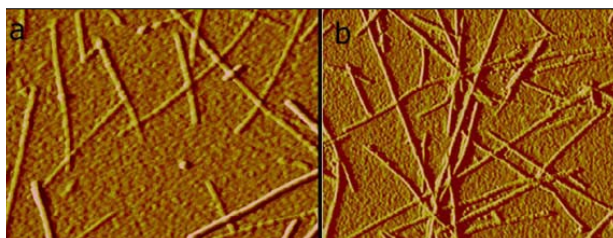


Figure 3. AFM image of amyloid fibrils of **a**) stefin A and **b**) stefin B (courtesy of M. Škarabot and I. Muševič).

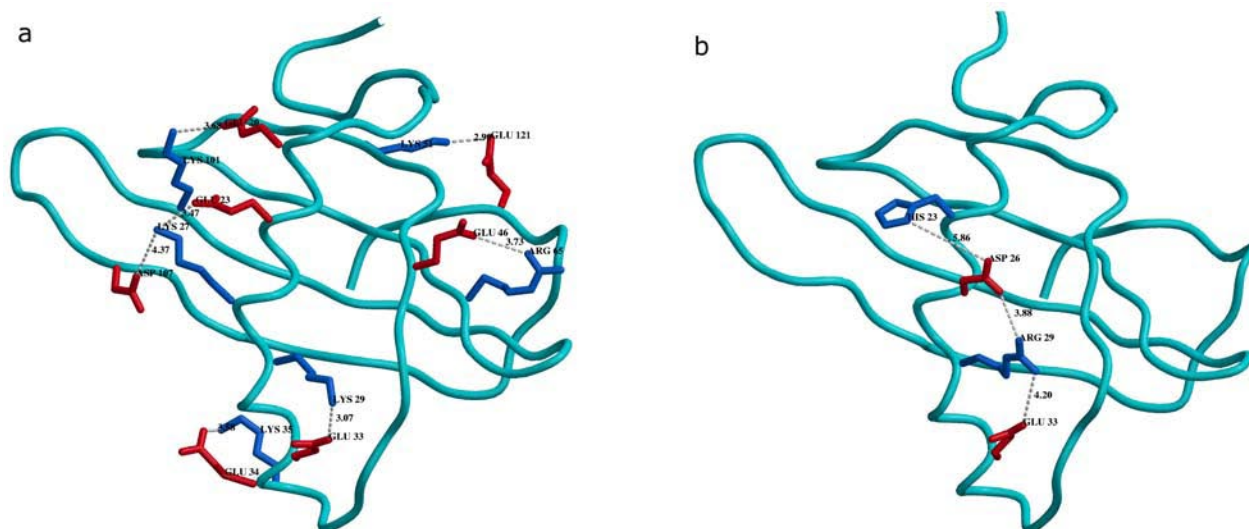


Figure 4. Different propensity to form amyloid fibrils results from the arrangement of specific salt bridges, which fix α -helix to β -sheet in **a)** stefin A monomer in contrast to **b)** stefin B. The figure was prepared with the MAIN program⁴³ (Turk 1992) and rendered with Raster3D program.⁴⁴

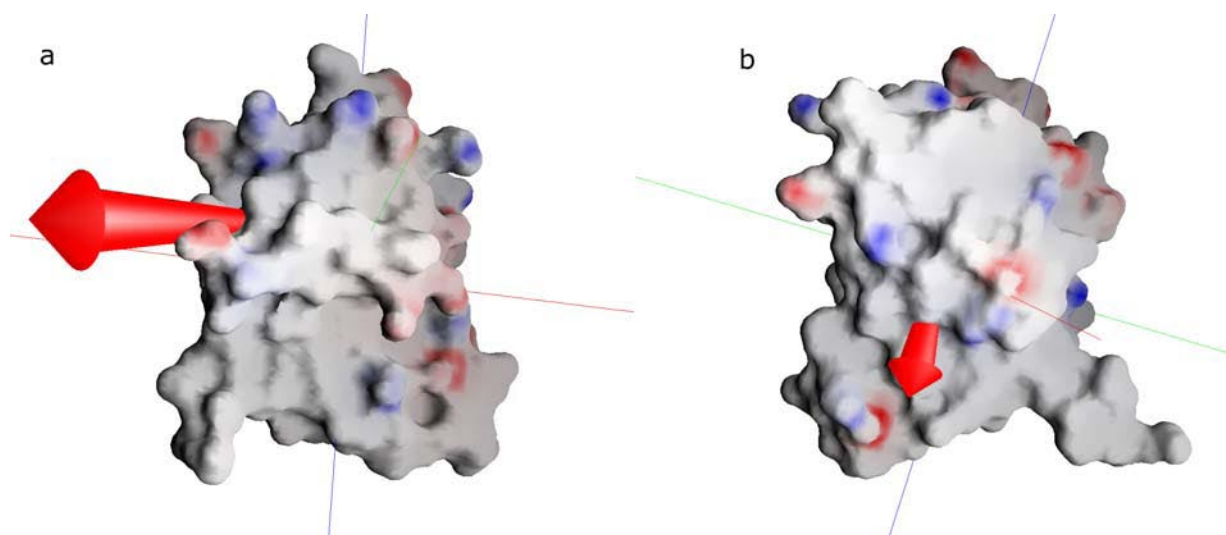


Figure 5. The surface charge distribution of **a)** stefin A and **b)** stefin B was calculated with Grasp program.⁴⁵ Stefin B is rotated 180° relative to the standard orientation. Negative charge is represented in red and positive charge in blue. The dipole moment vector is represented as red arrow.

To explain the difference in the propensity to form amyloid fibrils, structural analysis of the three-dimensional structures of stefin monomers and domain-swapped dimers was performed.⁴² The results suggest that major difference in stability of the homologues results from the arrangement of specific salt bridges, which fix the α -helix to the β -sheet in the stefin A monomer (Figure 4) and dimer. Easier detachment of the α -helix from the β -sheet could explain why stefin B forms amyloid fibrils already under very mild conditions in contrast to stefin A, which requires much more rigorous conditions.

To elucidate which additional factors influence the mechanism of fibrillation, we studied the distribution of the surface net charge of stefins and their mutants and the correlation between the dipole moment and the propensity for fibrillation.⁴² By using computer program Grasp⁴⁵ we calculated the electric dipole moment for stefins A and B⁴² and some stefin B variants/mutants, whose propensity for fibrillation has been measured. Stefin B S3Y31 variant and stefin B S3E31 variant with lower dipole moments (71 Debye and 86 Debye) have higher propensities for amyloid fibril formation than stefin A (146 Debye) and stefin B S3R4E31 mutant

(164 Debye) (S.J.K. and E.Ž., unpublished data). We therefore conclude that, at least in our series, that dipole moment is inversely correlated to the propensity to form amyloid fibrils.

Conclusions

Both stefins A and B undergo amyloid fibrillation, although the conditions needed differ substantially. The final amyloid-fibrils of both proteins, nevertheless, share common structural features characteristic of other amyloid fibrils.⁴⁰

Although, different models of amyloid fibril formation have been suggested, a generic mechanism is likely.¹⁵ Two homologous proteins, human stefins A and B, are therefore useful as model proteins in our studies of the mechanism of amyloid fibril formation. The most suitable model for the case of stefins seems to be the “domain-swapping” model. It has been shown that cystatin C and stefin A form domain-swapped dimers.^{22,23} It is not out of question that such a model could hold for many other amyloidogenic proteins, of which quite a number were shown to form domain-swapped dimers. The dipole assembly model of Xu et al.²⁴ is also supported by our case. A possible common model could thus represent a combination of the two: domain-swapped oligomers would chain-up due to increase in the dipole moment.

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Povzetek

Molekularni in celični mehanizmi nevrodegenerativnih bolezní, kot so na primer: Alzheimerjeva in Parkinsonova bolezen, amiotropna lateralna skleroza in nededne oblike prionskih bolezní, so verjetno skupni. Eden od pomembnih mehanizmov nevrodegeneracije je napačno zvijanje proteinov, ki agregirajo in tvorijo izvenselične plake (lehe) ali znotrajcelične vključke. Pričakujemo lahko, da bi natančnejše poznavanje mehanizma proteinske agregacije in posledične toksičnosti za celice vodilo do bolj racionalnega načrtovanja zdravil. Opisali bomo naše študije človeških stefinov A in B, ki služita kot dober model za amiloidno fibrilacijo. Študije obsegajo ugotavljanje mehanizma, slikanje fibrilarnih in prefibrilarnih delcev z metodama transmisijsko elektronsko mikroskopijo (TEM) in mikroskopijo na atomsko silo (AFM), in strukturne študije prekursorskih oligomerov.