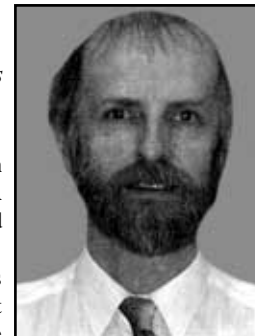# Protein Misfolding in Disease: Cause or Response?

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Abstract: Misfolding of newly formed proteins not only results in a loss of physiological function of the protein but also may lead to the intra- or extra- cellular accumulation of that protein. A number of diseases have been shown to be characterised by the accumulation of misfolded proteins, notable examples being Alzheimer's disease and the tauopathies. The obvious inference is that these proteinaceous deposits are pathogenic features of the disease. However, systems such as the unfolded protein response and ubiquitin-proteasome complex are in place in the cell to target misfolded proteins for degradation and clearance. Evidence suggests that in disease states, these protein-handling systems may be overwhelmed and the misfolded proteins accumulate as either extracellular deposits (eg. senile plaques in Alzheimer's disease) or intracellular inclusions (as in Lewy bodies in Parkinson's disease). These accumulations may be the direct cause of the particular pathology associated with the diseases or they may be inert "packages" designed to protect the cell from toxic insult.

INTRODUCTION

The "Conformational Diseases", as proposed by Carrell and Lomas [1], represent a diverse group of conditions characterised by protein misfolding, followed by self-association and subsequent deposition of the aggregated protein in the affected tissues. These deposits are usually fibrillar and have characteristic structural and histological morphologies while the biological effects of these differing protein deposits are distinctive and depend upon such features as the tissue involved and whether the deposits are intra- or extracellular. In the case of neurodegenerative diseases, the gross histopathological consequences of protein misfolding are features such as senile plaques and neuro-fibrillary tangles in AD, Lewy bodies in PD and Lewy body dementia plus other nuclear inclusions in the polyglutamate repeat diseases such as Huntington's disease and the ataxias. Similar inclusions may also be present in peripheral protein misfolding disorders eg. Mallory bodies in the liver.

1. THE DISEASES

There are large numbers of disorders which fall into the "conformational diseases" classification (Table **1**). Many of the diseases shown in Table **1** can be further subdivided (eg. hereditary inclusion body myositis [2,3]). The diseases discussed in this present review represent a few illustrative examples that hopefully contribute to our overall under-standing of protein misfolding disorders.

1.1. Alzheimer's Disease

The pathological hallmarks of AD are extracellular senile plaques, where the major component is the ABeta (Aβ)

peptide and intracellular neurofibrillary tangles (NFT's) comprising paired helical filaments, with hyperphosphoryl-ated tau representing the major constituent. All AD patients have many amyloid plaques containing degenerating nerve endings; their plaque count far exceeds that found in the normal ageing brain and the amyloid plaque load in specific regions of the brain correlates with the degree and type of mental impairment [4].

AD can be divided into early onset, familial AD (FAD), where sufferers develop the disease before the age of 60 years and late onset AD, where the age of onset of the disease is later than 60 years of age. Early onset AD is often synonymous with genetic AD, where the disease is inherited in an autosomal dominant manner. There is considerable confusion in the literature regarding the relative proportion of familial AD to 'sporadic' AD, where a single predominant genetic predisposition is absent, but where there may be genetic risk factors. To date, 3 genes have been identified as being linked to the disease - amyloid precursor protein (APP), presenilin-1 (PS-1) and presenilin-2 (PS-2). Polymorphic variants of a further gene - apolipoprotein E (ApoE) - have been shown to be a significant risk factor for late onset AD. Down syndrome patients (with 3 copies of the APP gene), who invariably develop classical AD pathology by age 50, produce high levels of Aβ from birth and begin to get amyloid plaques as early as age 12, long before they get tangles and other AD lesions [3]. Other genes have been claimed to confer risk of suffering AD, although these have not been confirmed in all populations, and therefore their significance is in doubt (e.g. α2-macroglobulin, α1-anti-chymotrypsin, ACE [5,6]). The genetics of PS-1 and PS-2 have been discussed in some detail elsewhere [7].

The FAD mutations of APP are clustered around the α, β and γ secretase cleavage sites and generally induce a common phenotypic change in the processing of APP resulting in an overall increase in the Aβ peptide or in the ratio of Aβ42 to Aβ40 [8]. This would appear to be of

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Table 1.   Proteins Susceptible to Misfolding and their Associated Diseases

Protein	Disorder
ABeta protein	- Alzheimer's disease - Dutch, Flemish, Italian cerebrovascular amyloidoses
tau protein	progressive supranuclear palsy, progressive subcortical gliosis, Pick's disease, FTDP-17, dementia pugilistica, arghyrophilic grain degeneration
-synuclein	Parkinson's disease
Prion proteins	CJD (familial, iatrogenic, sporadic); new variant CJD; GSS disease, fatal familial insomnia, sporadic insomnia, kuru
polyglutamate	Huntington's disease, spinocerebellar ataxias, dentato-rubro-pallido-Luysian atrophy, Machado-Joseph atrophy, spinobulbar muscular atrophy
Superoxide dismutase	amylotrophic lateral sclerosis
ABri/ADan	British/Danish dementia's
transthyretin	senile systemic amyloidosis
GFAP	Alexander disease
ATP7B	Wilson disease
haemoglobin	sickle cell anemia, unstable hemoglobin inclusion-body hemolysis, drug-induced inclusion body hemolysis
CTRF protein	cystic fibrosis
AH and AL (immunoglobin heavy and light chain fragments), AA (amyloid A), A 2M (beta2-microglobulin), ACys (cystatin C), ALys (lysozyme), AFib (fibrinogen A fragment)	immunity/inflammation disorders
IAPP, ANF fragment, calcitonin fragment, insulin	endocrine disorders
ApoAI and ApoAII, gelsolin, lactoferrin, lactadrehin	other systemic disorders

significance in AD pathogenesis since A 42 is the predominant species in the senile plaque [9]. With APP, it is not difficult to imagine that mutations near to the secretase sites may alter the proteolytic processing of APP. How the presenilin mutations mediate their effects is not known. Given the genetic evidence to date, the aetiology of the disease is currently best explained in terms of the amyloid hypothesis, which is supported by many key opinion leaders in the field. The basic tenet of this hypothesis is that the deposition of amyloid peptide in the parenchyma of the brain leads to neuronal loss and associated dementia. Interestingly, mutations within the A fragment (rather than close to the - and -secretase sites) usually result in diseases characterised by cerebral haemorrhage and/or vascular angiopathy (see [10]). Some of these mutations may increase the propensity of A to aggregate [11] although the Dutch (E693Q [12]), Italian (E693K [13]) Flemish (A692G [14]) and Iowa (D694N [15]) mutations do appear to have affects on APP processing [16]. Exactly what property imparts the vascular features is unclear.

There are over 100 known mutations to PS-1 which are generally found in exon 8 of the gene. The precise function of PS-1 is unknown although the protein is involved in the wnt/frizzled signalling pathway and possibly in the unfolded protein response (UPR) [17]. The most convincing phenotypic change mediated by the mutations in both PS-1 and PS-2 is an increase in the production of A 42, demonstrated in both transfected cells [18,19] and in transgenic mice [18,20,21]. It is not clear whether there is any relationship between A misfolding and PS-1 and the UPR. The presence of PS-1 mutations appears to lead to deficits in the function of the unfolded response leading to accumulation of unfolded proteins and apoptosis [17]. Defects in protein ubiquitination have been reported in AD, although not specifically linked to PS-1 FAD mutations [22].

Genetic linkage studies also identified an AD susceptibility locus on chromosome 19 that was subsequently shown to be the ApoE gene [23]. In man, three polymorphisms exist for the ApoE gene and population studies have demonstrated an increased frequency of the E4 isoform in AD patients [24]. Furthermore, there is a dose-dependent relationship between the number of copies of the E4 gene and the age of onset of AD such that E4/ E4 homozygote subjects have an earlier age of onset than heterozygous individuals [25].

Possession of the Apo E4 allele results in an increased amyloid burden in the brain, although how this is mediated remains to be elucidated and it is unclear how Apo E4 is associated with the increased risk. The risk associated with possession of the E4 allele has also been extended to patients with head injury [26] and intracerebral haemorrhage [27] and E4 patients with head injuries have a ten fold increased risk of developing AD [28].

1.2. Tauopathies

Tau is a microtubule-associated protein, a number of which function to stabilise tubulin dimers in the consolidation of stable axons and dendrites. Microtubules also have a critical function in cell division and intracellular trafficking where they serve as tracks for the transport of organelles such as mitochondria and synaptic vesicles and for the cell's disposal systems, such as aggresomes and phagosomes. The direction of cargo movement is under the control of dynein-kinesin motors and the over-expression of tau can inhibit organelle transport (see section 2.2).

An increased interest in tau has arisen through the identification of families with a mutation in the tau gene leading to FTDP-17, historically called Pick's disease [29,30]. These cases have inclusions within neurons that react with antibodies to tau, although these are not typical NFTs. Polymorphisms within the tau gene also appear to be risk factors for Parkinson's disease (PD), corticobasal degeneration and progressive supranuclear palsy [30,31]. Mutations in the tau gene perturb interactions between the tau protein and microtubules and may also increase the aggregation of the protein [32]. Despite the recognition that the presence of NFT's is a major pathological feature of AD, there are no proven genetic linkages between tau and the disease, although certain mutations in the tau gene can lead to fronto-temporal dementia with Parkinsonism or FTDP (see below). Furthermore, transgenic mice bearing the P301L tau mutation show tangle-like inclusions within 18 days of the injection of fibrillar Aβ1-42 into the cortex and hippocampus [33] and double mutant mice expressing both P301L tau and APPswe transgenes exhibit enhanced tangle pathology [34]. Thus, tangle formation may be secondary to amyloid deposition in AD. However, it may still play an important role in AD since it is generally admitted that the amount of NFT pathology correlates much more strongly with functional deficit than does Aβ pathology.

Tau-depositing disorders can be split into primary tauopathies where tau deposition is the first pathology (eg. amyotrophic lateral sclerosis (ALS)/PD-Guam, corticobasal degeneration, dementia pugilistica, Pick's disease) and secondary tauopathies, where tau deposition is a response to some other pathogenic stimuli or feature (eg. AD, Down syndrome, prion disease, multiple system atrophy (MSA), Nieman-Pick disease). In both primary and secondary tauopathies, however, the tau deposition may be necessary for degeneration to occur. In a number of cases, tau co-exits in deposits with α-synuclein (PD, Lewy body dementia, MSA, ALS) and there are very marked similarities between the two proteins. They are both (i) abundant neuronal proteins (ii) small, acidic, highly soluble, heat stable, ampipathic (iii) contain hydrophobic stretches of amino acids

(iv) have long half-lives (v) are phosphoproteins (vi) have point mutations in the genes which are autosomal dominant (vii) aggregation susceptible proteins where the aggregation composes the entire protein (viii) found in multiple neurodegenerative diseases (ix) are found in neuronal and glial inclusions (x) form 10-20nm diameter filaments (xi) are detected by Gallyas and thioflavin S staining (xii) form neuronal inclusions including neurofilaments, chaperones and other proteins, many of which are ubiquitinated and nitrated. *In vitro*, α-synuclein promotes tau aggregation and *vice-versa* [35]. This latter group also noted that mixed tau/α-synuclein aggregates occasionally formed and, *in vivo*, mice transgenic for mutant α-synuclein show inclusions of both α-synuclein and tau although it is not clear whether any functional pathology is associated with the expression of both proteins.

1.3. Parkinson's Disease

Parkinson's disease (PD) was first described by James Parkinson in 1817 in "An Essay on the Shaking Palsy". It is a neurodegenerative disease characterised by bradykinesia, rigidity and tremor (at rest) and pathologically by intracytoplasmic inclusions, known as Lewy bodies, in degenerating neurons of the substantia nigra. Idiopathic PD comprises >85% of all cases, the remainder being mainly neuroleptic-induced. It is rare for PD to occur before the age of 30 and the mean age of onset is over 60 years of age. In most cases, the cause of PD is unknown. The majority of cases are believed to be environmental in origin although no such factors have been conclusively identified. Less than 10% of all PD cases are known to be familial. The two major genes identified as being associated with familial PD, α-synuclein and parkin, are responsible for Lewy body positive and Lewy body negative forms of the disease, respectively. Autosomal dominant inheritance is rare although a few cases of mutations in the α-synuclein gene have been reported [36,37] and mutations in the parkin gene lead to autosomal-recessive juvenile Parkinsonism. The genetics of PD has been reviewed elsewhere [38-40]. The functions of α-synuclein and its β, γ and synoretin homologues are unknown although the α−, β−, and γ- forms have all been linked to neurodegenerative disease [41]. Mutations in the α-synuclein gene associated with familial forms of the disease probably increase the rate of oligomerization of the protein and lead to deposition in Lewy bodies [42]. α-Synuclein has been suggested as having a role in dopaminergic transmission, not only because of its relationship to PD but because it has effects on tyrosine hydroxylase activity and the dopamine transporter [43]. Furthermore, transgenic *Drosophila*, overexpressing human mutant α-synuclein exhibit a loss of dopaminergic neurons and develop motor dysfunction and α-synuclein-containing intraneuronal Lewy body-like inclusions [44].

The α-synuclein protein comprises an amino terminal region composed of amphipathic α-helical domains, a hydrophobic central core region and a glutamate-rich acidic carboxy-terminal end, features that support an interaction with lipid membranes [45]. In solution, α-synuclein has an unfolded random coil structure. Interaction with phospholipids, however, results in an increase in α-helical content and, at high concentration, it adopts the β-sheet

structure characteristic of amyloid fibrils [46]. Both - and - synucleins inhibit phospholipase D2 activity *in vitro* [47]. Furthermore, the interaction of -synuclein with polyunsaturated fatty acids appears to promote the formation of highly soluble oligomeric forms of the protein that later lead to the insoluble aggregated forms characteristic of PD [48]. The rate of -synuclein oligomer formation is also enhanced for the familial A53T mutant form of the protein.

-Synuclein was originally identified as the precursor of the non-ABeta-component (NAC) of Alzheimer disease plaques [49,50] although this association is not supported by all laboratories [51]. Polymerization of -synuclein, as well as being enhanced by familial PD mutations [42], is also accelerated by oxidative stress linked to iron and Cu(II) [52], A and NAC [53,54] and to mitochondrial inhibitors [55]. Thus, many stresses that lead to the aggregation of the A peptide also enhance the polymerisation of -synuclein leading to potentially toxic oligomeric forms. The intracellular inclusions containing -synuclein also contain large amounts of ubiquitin and proteasomes and thus resemble the aggresomes of Huntington's disease [56]. It is not clear, however, whether accumulation of -synuclein in PD is a consequence of the inability of the proteasome to degrade the misfolded protein aggregate. Interestingly, -synuclein, both in aggregated and monomeric forms, appears to bind to the S6' subunit of the 19S cap of the 26S proteasome and thus inhibit proteasome function [57].

1.4. Prion Diseases

In the context of disorders associated with protein misfolding, the prion diseases, including Creutzfeldt-Jakob disease (CJD) and new-variant CJD, Gerstmann-Straussler syndrome and, in animals, BSE and scrapie are of considerable importance. The topic of misfolding of prion proteins has been reviewed recently in this journal and the reader is referred to a previous publication in this review series [58]

1.5. Trinucleotide Repeat Diseases

Repeat CAG trinucleotide expansions in the coding regions of a number of genes are responsible for a specific group of neurodegenerative diseases including Huntington's disease (HD) and a number of forms of spinocerebellar ataxia. Each of the polyglutamine diseases has a distinctive neuropathology, although there is a considerable degree of overlap. There is, however, no homology between the genes showing the CAG repeats, other than the repeats themselves. These diseases do not appear to be due to the loss of the normal function of these expanded proteins but rather result from the gain of a toxic function of these mutant molecules.

HD is a genetically determined neurodegenerative disease with a clinical presentation of choreiform movements and cognitive impairment in middle age (although 10% of patients develop symptoms in childhood). HD, as an example of a polyglutamine expansion disease (in fact, the most prevalent of these disorders) displays characteristic intracellular inclusions [59]. The formation of intracellular aggregates or intraneuronal inclusions is a common feature of most polyglutamine disease and appears to be central to the pathogenesis of the disorder although inclusions have also been observed in the cytoplasm, axonal processes and

dendrites. The inclusions are generally found associated with areas of pathology but are not exclusively limited to neurons at risk [60]. Monomeric polyglutamine is unstructured but, in a manner thought similar to how the A protein folds and aggregates in AD, the polyglutamine proteins form antiparallel -sheets held together by hydrogen bond linkage between main chain and side-chain amides - the Perutz "polar zipper" [61]. By acting as transglutaminase substrates, cross-linking of the polyglutamine occurs such that covalently bonded aggregates are formed leading ultimately to intracellular precipitation or deposition [62]. *In vivo* studies have suggested that short protein fragments with expanded polyglutamine sequences readily form inclusions. Although inclusions may not directly kill cells, evidence suggests that they render the cell more susceptible to apoptotic stimuli. When cell death has been observed, however, it does not correlate particularly well with inclusion formation [63,64]. It may be that inclusion formation in these diseases is a cellular defensive mechanism in a similar manner to aggresomes in Huntington's disease.

The hereditary ataxias may be grouped into autosomal dominant, autosomal recessive and X-linked disorders which in many cases are due to trinucleotide repeats. Dentatorubral pallidolysian atrophy (DRPA) is an autosomal dominant neurological disorder characterised by progressive dementia, epileptic attacks, cerebellar ataxia and choreiform movements. It is associated with a CAG repeat expansion in exon 5 of the DRPA gene where the number of CAG repeats increases from the 3 to 36 in normals up to as many as 90 in affected individuals. DRPA encodes the cytoplasmic protein atrophin-1 which may play a role in apoptosis [65]. Expression of a truncated form of the DRPA protein with expanded polyglutamine sequence leads to the formation of filamentous aggregates [66] although it is not clear whether intracellular aggregates of the expanded DRPA protein are neurotoxic [67].

The spinocerebellar ataxias (SCA) are autosomally dominant progressive neurodegenerative disorders. Several of these ataxias are associated with polyglutamine expansions of their respective ataxin proteins. The SCA-1 protein, ataxin-1, is found in neuronal nuclei; in SCA-1 affected individuals, polyglutamine-expanded ataxin-1 is found colocalized with ubiquitin-positive nuclear inclusions [68]. It has been demonstrated that the expanded ataxin-1 is inefficiently degraded by proteasomes although ubiquitination is normal [69]. Such mis-degradation may release cytotoxic polyglutamate domains [70]. Transgenic mice expressing mutant ataxin-1 develop cerebellar ataxia and Purkinje degeneration [71] but although intracellular inclusions containing the mutant ataxin were found in mouse neurons, the expression of polyglutamine-expanded ataxin-1 in transfected cell lines did not result in cell death [68]. However, although the presence of intranuclear inclusions is associated with SCA-1 pathogenesis [64], transgenic mice expressing a mutant form of polyglutamine-expanded ataxin-1 where nuclear aggregates did not form nevertheless exhibited ataxia and Purkinje pathology which argues against an aggregate-dependent pathology [72].

SCA-3, also known as Machado-Joseph disease, is the most common of the hereditary ataxias with progressive

degeneration of spinocerebellar tracts. The associated intracellular protein, ataxin-3, is of unknown function. Polyglutamine-expanded mutant ataxin-3 can have up to 84 CAG repeat units, compared to a maximum of 37 in normal individuals [73]. Intracellular aggregates have been observed in transgenic mice expressing a truncated SCA-3 cDNA [74] and expression of polyglutamate-expanded full-length ataxin-3 in neuronal cells resulted in the formation of intranuclear inclusions and cell death [75]. SCA-6 involves mainly degeneration of Purkinje cells and is associated with a protein component of the alpha1A-voltage-dependent calcium channel [76]. It is believed that the polyglutamine expansion affects the normal function of the calcium channel, particularly in the cerebellum where expression in the highest [77]. The SCA-6 gene is therefore unusual (compared to the other ataxias) in that it encodes for a membrane protein. The precise consequence of the expansion is unclear although cells transfected with SCA-6 exhibit perinuclear aggregates and apoptotic cell death [78].

Expansion of the protein ataxin-7 is associated with SCA-7, where the expansion can contain up to 200 CAG repeats [79]. Although intranuclear inclusions of mutant ataxin-7 have been observed in pathologically affected areas, other non-affected areas, such as the cerebral cortex, also exhibit inclusions [80]. Furthermore, cells transfected with mutant ataxin-7, although showing accumulation of intranuclear inclusions, did not demonstrate evidence of cell toxicity [81]. Two other ataxias, SCA-2 and SCA-12 also display expanded CAGs but the presence of aggregates and/or inclusions is yet to be demonstrated.

The genetics and biochemistry of HD has been discussed in depth elsewhere in this volume [59]. It is believed that aggregation of the huntingtin protein results in oxidative stress and mitochondrial dysfunction that ultimately lead to cell death. However, protein aggregation need not be pathogenic and in some cases the aggregates may be beneficial in trapping non-functional mutant proteins thus reducing toxicity [82].

1.6. Motor Neuron Diseases

The motor neuron diseases are a heterogeneous group of acquired or inherited diseases characterised by motor cell death frequently leading to muscle wasting. In ALS, both upper and lower motor neurons are involved to varying degrees giving rise to a variable presentation depending on relative extent of involvement. The cause of ALS remains elusive despite the passage of more than a century since its first description by Charcot. A great variety of aetiological and pathogenetic mechanisms have been proposed. Over 90% of cases of ALS occur sporadically [83] where neither the cause nor the specific mechanism of the motor neuron loss has been defined. Various theories have been proposed including glutamate toxicity, free radical damage/oxidative stress and autoimmunity. In the 5-10% of familial cases, the disease appears to be associated with the superoxide dismutase-1(SOD1) gene on chromosome 21 where around 50 different mutations have been identified [84]. These mutations do not generally appear to impair dismutase activity per se but may endow the SOD enzyme with a gain of function that is lethal for motor neurons.

Data from Stathopulos *et al.* [85] suggests that the mutations in SOD leads to an increased aggregation of the protein as the mutant proteins have decreased stability. Oligomerization of the mutant CuZnSOD protein has been proposed as a cause of the motor neuron degeneration in ALS [86] although it has also been claimed that the mutant enzyme promotes oxidative damage [87]. In a hypothesis analogous to that suggested for A neurotoxicity in AD, mutant CuZnSOD protein undergoes misfolding and oligomerization into high-molecular weight aggregates which, at some stage in their formation, are toxic to motor neurons. Pathological differences between AD and ALS are apparent in that in the latter disease the aggregates form intracellular inclusions, as opposed to the extracellular A plaques familiar to AD. It is not clear whether the oligomerization and oxidative damage hypotheses are mutually compatable such that CuZnSOD oligomers cause oxidative damage or whether they bind to other intracellular proteins thus interfering with their normal function in the cell. However, there is no direct evidence that mutant CuZnSOD oligomers are neurotoxic and the CuZuSOD aggregates may also be an end-product of an over-loading of the cell's defence mechanisms (see section 2.1) where the cell is simply over-loaded with misfolded protein.

1.7. Familial British and Danish Dementias

These two rare autosomal dominant neurodegenerative disorders share pathological features of AD including amyloid plaques, NFTs, astrocytosis, microgliosis, neurodegeneration and progressive dementia. In Familial British Dementia (FBD), biochemical analysis of the amyloid plaques has identified a 4kDa peptide now known as ABri [88]. Some, but not all, deposits have been shown to be Congo red positive [89] suggesting the presence of -sheet structure. In FBD patients, the wild-type BRI precursor protein gene has a single nucleotide transition in the stop codon that allows read through and increases the length of the precursor protein from 266 to 277 amino acids thus leading to the appearance of the 4kDa ABri peptide [90]. In Familial Danish Dementia (FDD), the BRI precursor protein has a ten nucleotide duplication immediately before the stop codon. This results in a frame shift and a completely different C-terminal peptide that has been given the name ADan. Both ABri and ADan are, therefore, generated from mutations in the precursor BRI protein. They both share the same 22 amino acid N-terminal fragment although they also exhibit N-terminal heterogeneity [91]. It has been proposed that small non-fibrillar oligomeric species of ABri and ADan are the most potent pro-apoptotic forms of the peptide [92] (and El-Agnaf, personal communication). Interestingly, the amyloid plaques in FBD are a mixture of Congo red positive and Congo red negative forms while most plaques in FDD are negative for Congo red staining which may support the role of non-fibrillar forms of the ABri and ADan peptides in the pathology of these diseases [89] (and El-Agnaf, personal communication). Immunolabelling of brain tissue from FBD patients demonstrates tau pathology principally in the limbic system and is associated with NFTs and neuropil threads [89]. However, the absence of neurodegeneration in other NFT-affected regions suggests a lack of causal relationship. Moreover, the presence of hyperphosphoryated tau that is

virtually indistinguishable from that observed in AD suggests that tau is not the driving force behind these diseases.

1.8. Inclusion Body Myositis

As examples of peripheral amyloidoses, sporadic inclusion body myositis (SIBM) and hereditary inclusion body myopathy (HIBM) are worthy of consideration if only because they are associated with the accumulation, in muscle, of A and tubulofilaments, similar to the paired helical filaments in AD. SIBM is characterised by vacuolated muscle fibres and lymphocytic inflammation [93,94]. Genetic abnormalities have yet to be linked to HIBM although the varying clinical phenotypes suggests that a number of genes may be implicated [93,94]. Clinically, both forms of the disease are characterised by progressive muscular weakness. The unusual feature of SIBM and HIBM is the intracellular acccumulation in muscle of A , PS-1, BACE1, BACE 2, phosphorylated tau and Apo E [95,96]. Transfection of human muscle with APP results in A accumulation and HIBM-like pathology [97] supporting the link between overexpression of the precursor protein, upregulation of the APP processing system and cell necrosis. Furthermore, mice expressing a neutralizing antibody against NGF exhibit a muscular dystrophy and APP immunore- activity in myofibre cytoplasm [98] - these mice are also reported to display cerebral A deposits and neurodegene- ration [99]. It would thus appear that increased APP transcription and A accumulation may play a major role in the pathogenesis of these disorders. The precise cause of the cell death in HIBM and SIBM is unknown although choles- terol may play a role in the induction of A accumulation [100].

2. PROTEIN MISFOLDING - CAUSE OR SOLUTION?

In most cases, the critical event characterising the above disorders is the conversion of a native protein conformation typified by alpha-helix and or random structure, into - pleated sheet aggregates. In the neurodegenerative diseases (AD, PD, HD, ALS), at least, it is accepted by many that misfolding and deposition imparts a gain of function that ultimately leads to neuronal death [101] although there are opponents who challenge this dogma [102].

The general rule for misfolded proteins is that they are rich in -sheet structures, formed of alternating peptide pleated sheets [61]. In many cases, molecules of -sheet misfolded protein will polymerise into the fibril-type structures which characterise amyloid. The natural, active conformation of most proteins usually comprises a mixture of -helix and some unordered structure. In the pathological condition, the proportion of -helical structure diminishes with a concomitant appearance of highly -pleated sheet aggregates, characterised by a higher order fibrillar structure. Exceptions to this do apply with proteins such as transthyretin and 2-microglobulin, where the natural conformation is rich in -sheet, or tau aggregates which are composed mainly of -helices.

Amyloid fibrils appear as relatively straight, unbranched structures of 80-100 Å width and up to several µm in length. They are often seen to exhibit a helical periodicity consistent with a substructure of protofibers or filaments wound together [103]. The common property of the polymerised protein aggregates is proposed to be the induction of tissue damage, either by gaining a toxic activity or by losing the intrinsic biological function of the native protein. Attempts to conduct high-resolution studies of many of amyloidogenic proteins have been limited by their insolubility and non-crystalline nature. Studies by Serpell and colleagues [104] of A fragments and -synuclein have led to molecular models suggesting that amyloid fibrils are composed of a number of protofilaments exhibiting cross- conformation ie. hydrogen-bonding -sheet structure where the -strands run perpendicular to the fibril axis.

The conversion or misfolding of the protein is a product of one or many factors acting independently or in tandem. For instance, mutations in the native protein are one of the most common precipitators of misfolding although other factors such as ionic strength, pH, the presence of metal ions and the concentration of the protein itself can all play a crucial role. Much of our information on the regulation of amyloid formation is based upon studies of the A protein. It has been proposed that oligomeric seeds of A facilitate further protein misfolding, thus promoting polymerisation of the protein and eventual fibril formation [105,106]. Although a seeding process has not been demonstrated for the majority of other proteins susceptible to pathological misfolding, oligomeric intermediates have been reported for transthyretin, – synuclein and islet-amyloid polypeptide (iAPP) [107,108].

2.1. Ubiquitin-Proteasome System and the Unfolded Protein Response

Folding is part of the normal process that converts newly synthesised proteins to physiologically functional molecules. Up to a half of such proteins are handled through the secretory pathway where they are transported to the lumen of the endoplasmic reticulum (ER) for folding to occur. Folding proceeds through a series of folding intermediates. Misfolding can arise as a result of genetic mutations, mistranslation, environmental factors, changes in redox state, pH, to name but a few. The folding/misfolding of such proteins is generally controlled by molecular chaperones that facilitate normal folding (see [109] for review). Cellular proteins that fold incorrectly have potential to induce cellular damage. These proteins can be targeted for degradation by the UPS [110]. The accumulation of misfolded proteins within the endoplasmic reticulum (ER) results in a highly specific UPR [111] which, when activated, leads to either a reduction in ER stress or to apoptotic cell death [112]. Many studies of neurodegenerative diseases have demonstrated a close relationship between neuronal death, the ubiquitin system and the occurrence of ubiquitin-positive aggregated proteins. Similar ubiquitinated proteins are also found in the periphery in disorders such as inclusion body myositis [113] and in post-poliomyelitis muscular atrophy [114].

It is possible that under some conditions, these systems could be damaged or overwhelmed by the amount of misfolded protein such that there is an accumulation of ubiquitin-conjugated aggregates leading to neuronal dysfunction and ultimately to cell death. For instance, the

accumulation of iAPP in insulinomas is associated, both intra- and extra-cellularly, with ubiquitinated protein [115]. Furthermore, COS-1 cells transfected with human iAPP show accumulation of iAPP within the ER and Golgi where the rate of amyloidogenesis parallels that of apoptotic cell death [116]. Elements of the UPR are also found in SIBM [117] and many of the proteins accumulating intracellularly in muscle are those susceptible to misfolding, hinting at a causal relationship between protein aggregation and muscle atrophy. It is of note, however, that long standing denervation of muscle (post-poliomyelitis) results in tubulofilaments in muscle and Aβ deposits that are Congo red positive and immunoreactive to Aβ antibodies [114] - and these features are associated with cell death.

Although the characteristic extracellular Aβ plaques found in AD may themselves appear different to the intracellular proteinaceous inclusions found in PD, HD and other polyglutamate diseases, the second major pathological feature of AD are NFT's. These intracellular inclusions of abnormally phosphorylated forms of the microtubule-associated protein tau are found conjugated with ubiquitin. Moreover, there is evidence for Aβ oligomerization occurring intracellularly [118] and for its accumulation intraneuronally in AD [119] suggesting again that a cell seeks to segregate misfolded proteins in an attempt to protect itself but that this system is overwhelmed leaving a resultant Aβ deposit. It would also seem logical to assume that the ability of a cell to deal with aberrant proteins diminishes with age [120] and, indeed, the incidence of practically all conformational diseases increases with age. This may be a crucial factor in determining the late onset of many neurodegenerative disorders. Ubiquitination itself appears to become less efficient with ageing [121] and has been claimed to be defective in AD [22]. Furthermore, the frameshift ubiquitin mutant, ubiquitin+1, a product of molecular misreading, not only loses its capacity to ubiquitinate proteins but even appears to block the proteasome [121]. Ubiquitin+1 has been shown to accumulate in neurons in AD brain; the overexpression of the mutant ubiquitin induces apoptosis [122] and it has been proposed as a possible factor in the pathogenesis of AD [123].

A number of proteins linked to neurodegenerative diseases have effects on the UPR or UPS. Parkin reduces aggregation and cytotoxicity of an ataxin-3 fragment protein in an over-expression system and reduces the proteasome impairment arising from expression of the expanded polyglutamate protein [124]. Mutant PS-1 affects ER-stress inducers by down regulating the ER-resident chaperones of the UPR, such as the glucose regulated proteins (GRPs) [125] which are, for instance, known to bind APP and decrease Aβ secretion [126]. Furthermore, parkin functions as a ubiquitin E3 protein ligase, the protein that mediates in the conjugation of ubiquitin molecules to protein substrates [127,128]. Thus, a gain of aberrant PS-1 or loss of parkin function may impair proteasome activity leading to the accumulation of cytotoxic proteins. In addition to α-synuclein and parkin, a third gene, that encodes ubiquitin C-terminal hydrolase (UCH-L1) has been shown to be associated with familial forms of PD [129]. UCH-L1 is a de-ubiquitinating enzyme that is particularly abundant in the brain. The possible relationships between protein misfolding,

the UPS and UPR, particularly as they might operate in AD is shown in Fig. (**1**). The basic tenet is that cell stresses, be they oxidative, mutations or purely physiological can switch the folding of newly synthesised proteins from a native functional conformation to a misfolded state. The cell attempts to deal with these misfolded proteins by the UPR and UPS. However, various factors can result in the cell's defence machinery becoming overwhelmed such that aggregates and inclusions form - and somewhere along these pathways, cytotoxic species of misfolded protein are formed. It is also possible that cell stresses may have a direct effect on these protein handling systems. Finally, one may speculate that the ultimate sacrifice which the cell makes in such cases is to enter an apoptotic state although in AD there is little evidence for apoptosis other than for some initiator caspases to be activated.

2.2. Aggresomes

When the capacity of the proteasome system to degrade misfolded proteins is overwhelmed, aggregation occurs and proteins are moved to a ubiquitin-rich structure termed the "aggresome" [130]. As noted above, aggresomes form part of the cellular response to aggregated proteins and appear as inclusions in a number of protein deposition diseases. Aggresomes have been reported for SOD [131], parkin [132], α–synuclein [133], prion proteins [134,135] and cytokeratin-8 [136]. It is generally not clear whether aggresome formation is causative or protective although data has suggested that they serve a cytoprotective function, facilitating the degradation of toxic proteins [137].

Accumulating aggregated proteins can be directed to inclusion bodies by dynein-dependent retrograde transport on microtubules [138,139]. Kinesin-dependent anterograde transport is responsible for the movement of organelles such as neurofilaments and APP vesicles along microtubules [140]. This transport may be altered by familial PS-1 mutations [141] and inhibition of transport by tau leads to the accumulation of cargoes in the cell body [142]. It is thus tempting to speculate that the accumulation of aggregated, misfolded, ubiquitinated proteins is facilitated by increased tau expression and by PS-1 mutations. The idea that APP might serve as a membrane cargo receptor for kinesin-1 [140] might suggest that familial APP mutations would also have effects on microtubule transport. Furthermore, it has been reported that mutations in kinesin in Drosophila cause a motor neuron disease-type phenotype and mutations in the kinesin protein KIF1beta result in Charcot-Marie-Tooth disease type IIa [143]. Moreover, chronic axonal transport blockade has been implicated in Lewy body formation in Lewy body dementia [144]. Hence, the overall conclusion would be a belief that mis-regulation of degradation of misfolded proteins leads to their accumulation and eventual cell death, a common mechanism in neurodegeneration.

It is possible that all diseases associated with protein misfolding could be due to the inherent toxicity associated with some form of aggregated protein [145]. However, the precise means by which misfolded proteins, particularly amyloid proteins, may impart a pathological sequelae is not fully understood. The accumulation of huge (often kilogram) quantities of amyloid in affected tissues and organs is often
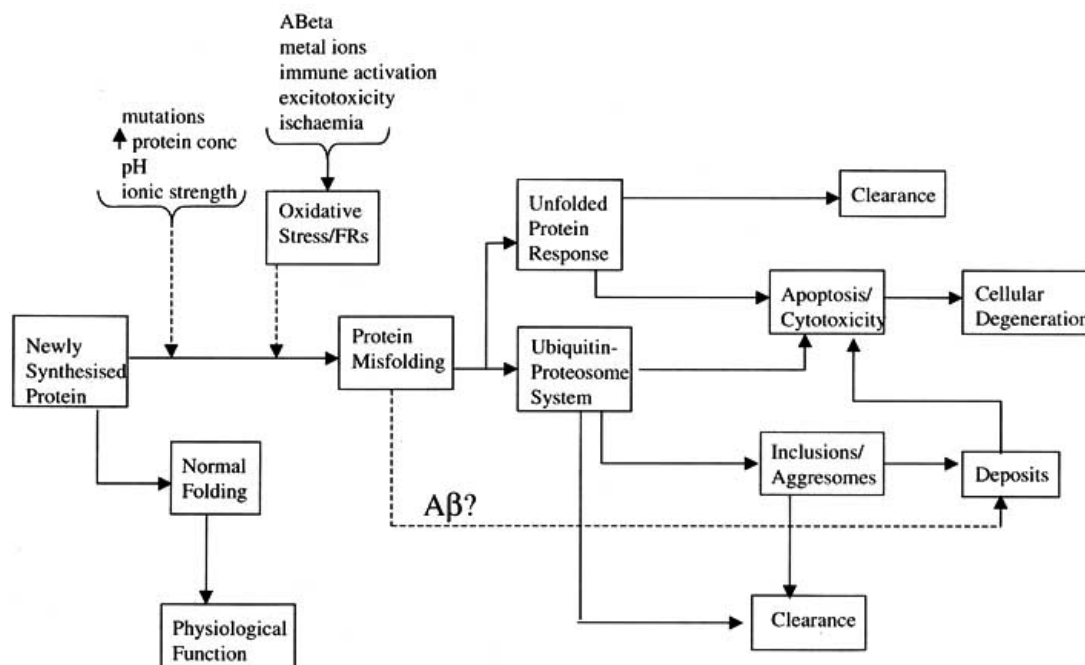
The Handling of Protein Folding/Misfolding within the Cell

Fig. (1). Newly synthesised proteins undergo folding through a series of intermediates, usually under the control of chaperones, in order to adopt physiologically functional conformations. A wide variety of stimuli either have direct effects on the folding process (eg. mutations in the proteins) or induce oxidative stress/free radical production, this leading to the generation of misfolded proteins. The cell attempts to deal with these proteins by systems such as the unfolded protein response (UPR) and ubiquitin-proteasome system (UPS). If these systems are overwhelmed, the resultant intracellular inclusions and extracellular deposits may induce cellular degeneration. However, the route to A deposits and the involvement of the UPR and UPS in AD is still unclear.

sufficient to cause the clinical symptoms in systemic amyloidoses. In neurodegenerative diseases, however, direct and specific interaction of the aggregated protein with the target cell may be the cause of the cell loss [146]. This interaction may involve (i) a loss of function, (ii) a gain of function or (iii) an inflammatory stimulus (for review see [58]). In immunoglobulin light chain amyloidoses, the folded protein deposits can occur in virtually any peripheral tissue. Suppression of light chain production results in functional organ recovery without the removal of the amyloid deposits suggesting that, as in AD, prefibrillar toxic oligomers may be the pathological agent [147]. Nevertheless, there is still considerable debate as to whether the deposits are a cause or function of the disease (see [148] and refs therein).

3. TRANSMISSIBILITY

Even prior to the BSE epidemic in cattle in the UK in the 1990's, the transmissibility of the prion diseases was appreciated through our knowledge of sheep scrapie and kuru. All of the so-called "transmissible dementias" (Creutzfeldt-Jakob disease, Gerstmann-Straussler-Sheinker syndrome, Kuru and spongiform encephalopathies in a number of animal species) are characterised by the conversion of a normal cell protein (the prion protein - PrP) into an abnormal isoform (PrP^{sc}).

Despite the biophysical similarities between prion protein nucleation and aggregation mechanisms and those reported widely for other amyloid proteins, little credence has been given to the possibility that, given the right vehicle, AD and other amyloidoses could be transmissible. Nevertheless, it was shown a decade ago that the injection of brain extracts from patients with AD intracerebrally into a marmoset resulted in the formation of amyloid plaques and dystrophic neurites [149]. A recent report by Lundmark and co-workers [150] has demonstrated the transmissibility of systemic amyloidosis in mice following oral ingestion of a splenic extract from animals with a chemically-induced amyloidosis and the injection of silk plus an inflammatory stimulus results in splenic amyloidosis in mice [151]. Conversely, although spider silk is a protein rich in -sheet structures [152] there is no evidence of any occupationally related diseases in silk workers [153]. Nevertheless, as well as BSE and sheep scrapie prion diseases being transmissible within the species, amyloid arthropathy is transmissible by aerosol in chickens [154]. All amyloidoses may, therefore, be intra-species transmissible, given the right vehicle and optimal conditions. It may be that cross-species transmission does not occur with all prions/amyloids - or it may be that we are just not aware of it, yet.

4. AGGREGATION INHIBITORS

Having long been a therapeutic target for many pharmaceutical companies, there are many reviews discussing inhibition of Aβ aggregation (eg. [155,156]) showing activity in a diverse range of chemical structures from small peptide-based molecules [157-159] to series such as benzofurans [160] and rifampicin-type molecules [161]. This diversity of chemical structures suggests that multiple binding sites may exist for inhibitors on the Aβ molecule and/or that the inhibitors are recognising a conformational state of the peptide. This latter suggestion would be supported by data showing that some Aβ aggregation inhibitors are also able to interfere with the folding of other amyloidogenic proteins. Probably the first compound shown to bind to multiple amyloids was Congo red that has been used for identifying amyloid in a variety of tissues for over half a century. Congo red itself has been shown to inhibit Aβ fibril formation and toxicity [162], huntingtin fibre formation [163] and to inhibit conversion of the prion protein to the protease resistant form [164]. Congo red administration also significantly disrupts *in vivo* formation of polyglutamine oligomers and significantly improves motor behaviour in the R62 transgenic mouse model of HD [165]. One of the first potential drugs exhibiting effective inhibition of aggregation was the anthracyline 4'-iodo-4'-deoxydoxorubicin (IDOX) which was claimed to reduce human immunoglobulin light-chain amyloidosis [166], to decrease PrP accumulation and improve survival time in Syrian hamsters innoculated with scrapie-infected brain homogenate [167] and to bind to amyloid fibrils of different chemical types [168]. The means by which IDOX recognises these amyloids is unknown although a structural model of IDOX docking with Aβ fibrils has been reported [169]. Similar tetracycline-type molecules have also been shown to be inhibitors of Aβ fibrillization [170], and to prevent aggregation and acquisition of protease resistance of prion protein peptides [7].

There has been some success in attempts to identify a binding site on Aβ for inhibitors in that a benzofuran specific site has been described [160]. However, binding to this site was not saturable, possibly due to the formation of peptide-inhibitor complexes. Interestingly, benzofurans of this series have also been shown to be inhibitors of transthyretin misfolding (Saraiva, personal communication), Similar conformation-specific recognition has been proposed with an antibody raised to soluble oligomers of Aβ and which is claimed to inhibit the toxicity of oligomers of α-synuclein, iAPP and polyglutamate proteins [171].

A series of NSAID's have been shown to be inhibitors of transthyretin amyloidosis and are believed to prevent the dissociation of the natural tetramer, thus preventing subsequent fibril formation [172]. It has also been shown that NSAID's prevent the β-sheet folding of human amylin [173]. NSAID's, such as ibuprofen and naproxen [174] and diclofenac, meclofenamate and diflusinal do inhibit Aβ 1-40 fibrillization, but only at concentations greater than 100uM (Howlett unpublished). Nevertheless, administration of NSAIDs, particularly ibuprofen, is associated with a decrease of up to 50% in the risk of AD [175,176]. Ibuprofen has also been shown to decrease plaque pathology in Tg2576 APP transgenic mice [177] and ibuprofen and naproxen are claimed to bind to senile plaques in AD brains, suggesting their use as imaging tools [174]. Although NSAIDs have been demonstrated as having neuroprotective effects in cell-based models of PD, these effects are probably a result of inhibition of oxidative stress [178,179]. There have been no reports of NSAIDs interacting with α–synuclein or other amyloidogenic proteins.

The antifibrillization effects described by Solomon *et al.* 1996 [180] and harnessed by Elan Pharma [181] provide an exciting concept where an immune response, rather than being generated against a microbe, is directed against self-antigens. Although effective in removing fibrillar Aβ in transgenic mouse models of Aβ deposition, human vaccination trials were halted due to unacceptable serious side-effects [182] and we have yet to see proof that the serious side effects observed in AD patients vaccinated with Aβ peptide were not an exacerbation of their disease state. Nevertheless, a recent report from a Zurich cohort of the Elan trial has claimed that antibody responders did exhibit a slowing in cognitive decline [183]. In theory a similar approach could be directed towards other diseases associated with neurotoxic antigens although the encephalomyelitis observed in the human Aβ trials may be a common phenomenon following self-antigen administration [184].

Data on inhibitors of other protein aggregates is much more sparse. The assembly of α–synuclein can be inhibited by β- and γ-synucleins [185] and it has been proposed that the use of anti-aggregatory β-synuclein-derived peptides could provide a new treatment for AD and PD [186].

5. SYNERGISM

At first sight, AD, PD, HD and prion diseases would appear to be discrete neurodegenerative disorders, characterised by a distinctive pathology. Similarly, in the periphery, familial amyloid polyneuropathy, aside from it being an amyloidosis, would appear to be a distinctive transthyretin-dependent disorder. However, not only do agents such as Congo red and IDOX bind to multiple amyloid types (as noted above) but the amyloids are able to associate with or maybe bind to each other.

Transthyretin has certainly been shown to associate with Aβ protein [187]. It may even prevent the Aβ protein from taking on an amyloid conformation [188] as it is elevated in Aβ plaque prone areas in young Tg2576 mice and is associated with the plaques when they do finally appear [189]. The presence of increased transthyretin protein in the brains of pre-plaque Tg2576 mice at a time when the CNS Aβ levels are already significantly elevated may suppress the aggregation and deposition process [189].

Another protein found co-localised with Aβ plaques in some cases of AD is the cystatin-C protein. A genetic variant of this protein, with an A68T mutation, is responsible for a condition known as hereditary cerebral haemorrhage with amyloidosis, Icelandic-type, characterised by amyloid deposition in both the CNS and in peripheral tissues. In the AD cases, however, there is no evidence for a mutated form of the cystatin-C gene to be present. Nevertheless, cystatin-C does appear to bind with high affinity to Aβ in senile plaques; the pathological consequences of this interaction remain to be established.

Despite the obvious disease-linkage and often close pathological proximity of tau and A in AD, evidence of a biophysical association is lacking. An association between tau and – synuclein has, however, recently been reported [35] suggesting that interactions between the two can promote fibrillization and the subsequent formation of intracellular inclusions. The accumulation of – synuclein in intracellular inclusions is a characteristic of LBD and most cases of sporadic and familial AD do develop Lewy-body like inclusions [190]. Furthermore, Down syndrome patients, where early AD-like symptoms are observed, also show – synuclein positive inclusions [191]. When lines of transgenic mice overexpressing hAPP and human – synuclein are crossed, the progeny have increased accumulation of – synuclein, enhanced cognitive impairment and neurodegeneration but show little change in plaque deposition [192]. This may suggest an effect of – synuclein on non-plaque-type A (ie. protofibrillar) mediated neurodegeneration. It has also been claimed that NACP binds to and promotes the fibrillization of [193]. There would appear little doubt, therefore, that there is some link between and – synuclein (or NAC) but whether that relationship is at the biophysical level is not yet clear.

Although mice overexpressing human mutant APP transgenes deposit plaques from 8-12 months of age, depending on the line, it is generally noted that there is little, if any, neurodegeneration in these animals [194,195]. Of course, it could be argued that human aggregates are not toxic in mouse brain: attempts to demonstrate neurotoxicity by direct intracerebral injection have certainly been problematic [196]. Alternatively, as noted above, the presence (eg. transthyretin or parkin) or absence (eg. – synuclein) of other factors may also play a role in determining the protein folding route taken by . Interestingly, although it is often commented that "mice do not get Alzheimer's disease", deposits of rodent are observed in NGF auto-antibody mice [99] and rodent is found associated with human deposits in human APP transgenic mice (Howlett, unpublished).

CONCLUSIONS - ARE THERE ANY?

Undoubtedly, a large number of human diseases are associated with the accumulation of a whole myriad of misfolded proteins. This accumulation can be in tissues, intra- or extracellularly, in the CNS or in the periphery and contained within inclusions, aggresomes or filaments. Overall, the currently available evidence does not strongly support the theory that these protein accumulations serve any pathogenic function. Rather, the evidence shows that in many cases the accumulations are the body's attempt to deal with what appear to be foreign proteins. This is not to say that the earliest species of misfolded proteins, before they are dealt with by the UPR, UPS or other protein-chaperoning devices are not cytotoxic. In fact, as has been reported by Walsh and colleagues in this issue [146], evidence does support a pathogenic role for small oligomeric forms of the peptides although conclusive proof of even this is lacking. Until we have such proof, which will probably only come from further trials in patients of potential disease-modifying therapeutic agents, the jury will remain "out" on the precise role of aggregates and inclusions in human disease.

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