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Proteostasis and Secondary Proteinopathy in Alzheimer's Disease

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Abstract

It is now widely recognized that the brains of Alzheimer's patients often display multiple pathologic abnormalities, termed mixed proteinopathies. These individuals will by definition always have amyloid pathology mixed with neurofibrillary tangles, may also have TDP-43 or α-synuclein pathology. The basis for the preponderance of mixed pathology in Alzheimer's Disease (AD) is poorly understood, but recent studies have suggested that compromised function of the proteostasis network could be an important contributing factor. The term proteostasis network refers to the myriad of activities and functions that work in concert to maintain the proteome. In settings of neurodegeneration, it is thought that high levels of misfolded proteins produce an added burden on the proteostatic network by occupying various activities required to dissociate such aggregates and degrade the misfolded proteins, leaving vulnerable "by-stander" proteins at greater risk for misfolding and aggregation. In proteomic studies of brains from mice with high levels of Alzheimer-amyloidosis, we have recently determined that a number of cytosolic proteins solubility as amyloid burdens rise. This finding is consistent with the hypothesis that amyloid deposition can, by some manner, impinge on the function of the proteostatic network to cause "secondary" misfolding. Thus, in mice that model human neurodegenerative pathology, evidence is emerging in support of the concept that the accumulation of one misfolded protein can, by some manner, impact on the folding of others.

Keywords: Alzheimer's; Proteinopathies; Amyloid pathology; Neurofibrillary tangles; TDP-43 inclusions

Introduction

The proteostasis network is thought to be delicately balanced in the sense that the levels of protein synthesis, chaperone activities, and degradation activities (proteasome and autophagy) have co-evolved to optimize the use of energy resources and regulate the abundance of critical regulatory factors [1]. In essence, the components of the proteostasis network within cells are proposed to be present in sufficient but not excess levels [2]. In this scenario, any insult that diminishes the function of, or adds burden to, one or more elements of the network could create a condition of proteostatic insufficiency. In such an environment, cells may be unable to correctly fold every newly made protein or efficiently degrade every damaged or misfolded protein. Thus, the accumulation of one misfolded protein could cause collateral, or secondary, misfolding by engaging and overwhelming various chaperone or degradative activities, leaving vulnerable by-stander proteins at greater risk of failing to achieve native structure. The concept of collateral misfolding was first elaborated in studies of invertebrate models expressing aggregation prone fragments of mutant huntingtin [3], and later by expressing mutant superoxide dismutase 1 (SOD1) [4]. In this invertebrate model system, the evidence that aggregation of mutant huntingtin, or SOD1, impaired the proteostatic network arose from the observation that proteins harboring temperature sensitive mutations could no longer achieve native conformation when the animals were cultured at the permissive temperature. This finding suggested that the activities responsible for folding these temperature sensitive proteins may have been diverted to the task of dis-aggregating and degrading the mutant huntingtin, or SOD1, causing the overall network to be overwhelmed by the constant influx of newly synthesized polypeptides. In relation to neurodegenerative disease, it has been argued that pathologic accumulation of intracellular proteinaceous aggregates is, in and of itself, an indication of an overburdened proteostasis network [1,5,6].

Alzheimer's disease (AD) has traditionally been defined neuropathologically as an amyloidosis with neurofibrillary tangles. In studies of individuals with dominantly inherited forms of AD, it has become clear that the deposition of amyloid in AD occurs well before the onset of cognitive symptoms and the appearance of NFT pathology [7,8]. It is now increasingly recognized that the brains of AD patients can display pathologic inclusions containing α-synuclein and TDP-43 in addition to the invariably found amyloid and NFT pathology [9-13]. For example, approximately 20-50% of AD cases present with TDP-43 inclusion pathology with tau and TDP-43 typically observed as separate entities within the cell body of any neurons containing aggregates of both proteins [12]. Similarly, in several pure tauopathies, such as corticobasal degeneration and Pick's Disease, the brains of affected individuals can also present with TDP-43 pathology [9-17]. Importantly, in mice over-expressing mutant tau we have recently demonstrated the coexistence of robust tauopathy with secondary TDP-43 pathology [18]. By virtue of the perceived order of appearance and the lack of direct genetic association with the primary disease, these pathologies could be viewed as "secondary" events in the evolution of these diseases. Thus, one could potentially explain the appearance of tau, α-synuclein, and TDP-43 pathologies in AD as secondary events that occur because the deposition of amyloid, by some manner, increases the burden on the proteostatic network to

create an environment in which tau, TDP-43, and/or α-synuclein become more vulnerable to misfolding.

Modeling Alzheimer-amyloidosis in mice

Multiple laboratories have created mice that express mutant amyloid precursor proteins, with and without co-expression of mutant human presenilin, as a means to model Alzheimer-related pathology [19]. Apart from focal abnormalities in tau adjacent to amyloid deposits, these mice do not progress to develop neurofibrillary tangle pathology or significant accumulation of tau aggregates [20-25]. In our experience, the vulnerability of endogenous mouse tau or TDP-43 to secondary misfolding in mice modeling Alzheimer amyloidosis is limited [20,26]. These findings are generally at odds with the notion that amyloid could be inducing a secondary misfolding of tau or TDP-43. Nonetheless, it is possible that the murine versions of these proteins are simply less prone to misfolding and aggregation.

To determine whether amyloid may impose any burden on the proteostatic network, we undertook a proteomic study to search for evidence of secondary protein misfolding in the APPswe/PS1dE9 mouse models of Alzheimer amyloidosis [26]. This recently published study determined that a number of cytosolic proteins lose solubility as amyloid accumulates. To identify proteins that lose solubility, we developed a protocol in which brain homogenates were sequentially extracted and centrifuged in detergents of increasing strength (NP40 →deoxycholate→ SDS) to separate soluble and insoluble proteins. This approach was first put into practice in a study to identify proteins in cultured cells that lose solubility upon heat stress [27]. Coupling detergent extraction and sedimentation protocols to LC-MS/MS approaches, we identified more than 20 cytosolic proteins that were specifically over-represented in the SDS-insoluble fractions of the brains of mice with high amyloid burden [26] (Table 1).

Gene Name (common name)	Expression Pattern*	Verified by immunoblot
ALDOA (Fructose-bisphosphatealdolase A)	Neurons only	
ARF1 (ADP-ribosylation factor 1)	Neurons only	
CLTC (Clathrin heavy chain 1)	Neurons only	
DNM1 (Isoform 3 of dynamin 1)	Neurons only	
ENO1 (Alpha enolase)	Neurons only	Yes
ENO2 (Gamma enolase)	Neurons only	Yes
PGAM1 (Phosphoglycerate kinase 1)	Neurons only	Yes
NSF(Vesicle fusing ATPase)	Neurons only	
STXBP1 (Isoform 1 syntaxin-binding protein 1)	Neurons only	
YWHAB (Isoform long of 14-3-3 protein beta/alpha)	Neurons only	
YWHAG (14-3-3 protein gamma)	Neurons only	
YWHAH (14-3-3 protein eta)	Neurons only	Yes
YWHAZ (14-3-3 protein zeta/ delta)	Neurons only	
ACO2 (Aconitatehydratase, mitochondrial)	Neurons and White Matter	
ACTC1 (Actin, alpha cardiac muscle 1)	Neurons and White Matter	
CKB (Creatine kinase B-type)	Neurons and White Matter	
DPYSL2 (Dihydropyrimidinase-related protein 2)	Neurons and White Matter	
EEF1A1(Elongation factor 1-alpha 1)	Neurons and White Matter	
GAPDH (Glyceraldehyde-3-phosphatase dehydrogenase)	Neurons and White Matter	Yes
GDI1 (Rab GDP dissociation inhibitor alpha)	Neurons and White Matter	
HSPA8 (Heat shock cognate 71 protein)	Neurons and White Matter	
HSP90AA1 (Heat shock protein 90-alpha)	Neurons and White Matter	
HSP90AB1 (Heat shock protein 90-beta)	Neurons and White Matter	
PGK1(Phosphogycerate kinase 1)	Neurons and White Matter	
YWHAE (14-3-3 protein epsilon)	Neurons and White Matter	
YWHAQ (Isoform 1 of 14-3-3 protein theta)	Neurons and White Matter	
APOE (Apolipoprotien E)	Astrocytes	
CKB (Creatine Kinase B-type)	Astrocytes	
CLU (Clusterin)	Astrocytes	Yes
EEF1A1 (Elongation factor 1-alpha 1)	Astrocytes	
*Expression pattern assessed by the Allen Brain Atlas (http://mouse.brain-map.org)		

Table 1: Characteristics of proteins that lose solubility in older APPswe/PS1dE9 mice

The over-representation of these proteins in SDS-insoluble fractions from the brains of older APPswe/PS1dE9 mice was partially verified by immunoblotting with antibodies to α-enolase (Eno2 or NSE), α-enolase (Eno1), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 14-3-3 eta and phosphoglyceratemutase 1 (PGAM1) (Table 1). As predicted by our previous work in this model[20], endogenous mouse tau was found only in PBS-soluble fractions by both LC-MS/MS and immunoblotting [26]. No insoluble forms of these proteins were detected in younger APPswe/PS1dE9 mice, aged 2 and 6 months, or non-transgenic (NTg) mice of any age. More recently, we have determined that we first observe cytosolic proteins to lose solubility in the brains of APPswe/PS1dE9 mice at about 12 months of age (not shown). At this age, amyloid burden has begun to build to levels similar to what would be observed in a typical early AD case. The age-dependent loss of solubility of cytosolic proteins in this model

is consistent with the notion that amyloid deposition, by some manner, impairs proteostatic function.

Although most of the proteins identified as losing solubility are expressed in neurons, it is difficult to determine which neurons are affected and in which subcellular compartments these "misfolded" proteins might be located. This question could be best addressed by immunostaining tissues from these mice with antibodies that specifically recognize non-nativelyfolded isoforms of these proteins, but to date reagents for this specific purpose have not been developed. It is possible that these proteins are localized primarily in dystrophic neurites surrounding mature amyloid plaques. Localization within these structures might mean that the decrements in proteostatic function are compartmentalized. Such sequestration might be inconsequential to cell function or might become a nidus of protein misfolding that spreads throughout the cell.

A concern in interpreting our findings is that it is difficult to demonstrate definitively that the loss of protein solubility in older APPswe/PS1dE9 mice is not a consequence of non-specific interactions between abundant cytosolic proteins packed within neurites and the amyloid fibrils that would be in close proximity. Importantly, the loss of solubility by normally cytosolic proteins in the older APPswe/PS1dE9 mice was not global in nature. There were still a large number of proteins that were found only in the PBS-soluble fractions, including abundant proteins such as endogenous tau, α-synuclein, and SOD1 [26]. These proteins would be expected to be found in neurites adjacent to amyloid deposits. To control for non-specific binding of cytosolic proteins to Aβ aggregates, we prepared homogenates of brain from older non-transgenic mice and mixed in pre-aggregated Aβ40/42 peptides before performing the detergent extraction and centrifugation protocol. Detergent insoluble fractions generated from these samples were then immunoblottedwith antibodies to the proteins listed in Table 1, finding none of the proteins fractionated with the aggregated Aβ in the insoluble fraction, and all were exclusively found in soluble fractions [26]. Although this effort represented the best control we could devise, further study is required to determine whether our observations demonstrate a failure in proteostatic function. For example, one prediction would be that enhancing proteostatic function should prevent the loss in cytosolic protein solubility. Alternatively, if the proteins we have identified have failed to achieve native conformations, one would expect that there would be loss of function in the pathways these proteins populate.

Using gene ontology analysis [Panther tools] to classify the proteins that lose solubility in the brains of older APPswe/PS1dE9 brains, the most abundant classes of proteins identified were chaperones (9 out of 28 proteins: 6 members of 14-3-3 family and HSP90AA1, HSPA8, HSP90AB1, p=1.68x10-9) and enzymes involved in glycolysis (6 out of 28 proteins, including GAPDH, ENO1, PGAM1, ALDOA, PGK1 and ENO2, p=1.35x10-7 by biological process, p=1.99x10-10 by pathway). Whether the loss in solubility of these proteins is sufficient to produce consequential deficits in functional pathways has not yet been established. In no case did we observe a complete loss of solubility for any of these proteins, thereby implying that any loss of functionality would be partial. Still, evidence of loss of function in relevant pathways, such as glycolysis, would provide evidence that corroborates the proteomic data.

Modeling AD secondary pathologies in mice

Most available evidence indicates thatthe deposition of Aβin human ADprecedes the appearance of abnormal tau [7,8]. A large body of work in transgenic mice had provided data to further support a causal relationship between amyloid deposition and the development of tau pathology [21-25,28-51]. However, one recent study of mice that co-express mutant APP, mutant PS1, and mutant tau (3x Tg-AD mice) indicates that the rate of mutant tau misfolding and aggregation can be independent of the influence of Aβ and amyloid [52]. One of the major gaps in knowledge for the AD field is a poor mechanistic understanding of how Aβ, Aβ oligomers, or amyloid deposition may trigger the secondary misfolding of tau and other "secondary" targets. Some recent studies have linked the binding of Aβ oligomers to the cellular prion protein PrPC as simulating Fyn kinase, which may directly or indirectly increase tau phosphorylation [36,40,53]. Thus, by one or more signaling pathways, some form of Aβ may alter the phosphorylation of tau thereby heightening its probability of misfolding. Once misfolding of tau begins, the altered conformation appears to be able to spread between cells [54]. By this scenario, the genesis of tauopathy could involve a multifaceted process in which Aβ, or amyloid, mediated signaling events induce changes in tau phosphorylation to enhance the probability of misfolding. Once tau misfolding takes hold in a specific brain region, then the pathology could spread throughout the CNS. Although there is evidence in some systems that one type of misfolded protein can "seed" the misfolding of another (e.g. a-synuclein and tau) [55], whether Aβ, Aβ oligomers, or amyloid fibrils can directly cross-seed tau misfolding remains uncertain. Based on our observation that high amyloid burden can trigger the loss of solubility of neuronal cytosolic proteins in mice, we suggest that disruption of proteostatic networks in neurons surrounded by amyloid could be contribute to the spreading or seeding of tau, α-synuclein, or TDP-43 proteinopathy. Thus, different forms of Aβ could act at multiple levels to induce tau misfolding and then facilitate the spread of these misfolded conformations throughout the CNS.

A question yet to be answered is whether the proteins we have identified as losing solubility in mice with high amyloid burden become insoluble because the proteostatic network has been negatively affected by the deposition of amyloid. It is conceivable that misfolded Aβ accumulating in the interstitial spaces could enter cells to directly impact the folding of cytosolic proteins. Engineered proteins with amyloid-like domains can interact with a number of cellular proteins [56]. In studies where Aβ peptide was expressed in the cytosol of the muscle lining the outer wall of *C.elegans*, the peptide produced abnormalities that are consistent witha capacity to diminish proteostatic function [57]. In mammalian primary neuron cultures, Magraneet al. observed that intracellular expression of Aβ produced defects in Akt survival signaling, and these abnormalities could be ameliorated by induction of the heat shock chaperones [58]. Thus, if misfolded Aβ accumulating in the extracellular space can penetrate membranes, then it could conceivably directly degrade proteostatic function.

Prospects for therapeutic intervention

If the evolution of secondary proteinopathies in AD, fronto-temporal dementia (FTD), and other neurodegenerative disorders is the result of proteostatic stress, then augmenting proteostatic network function should mitigate secondary misfolding. Based on numerous reports in the literature that have described positive benefits from life-

long treatment with rapamycin and other inducers of autophagy [59-64], inducing clearance mechanisms may be an efficacious therapeutic approach that could provide a substantial enhancement of proteostatic network function.

Manipulating inducible chaperone function in neurons in vivo appears to be challenging. Pharmacologic and genetic approaches have been used in numerous studies as a means to induce the expression of the inducible heat-shock chaperones. In culture, neurons have been shown to respond to Hsp90 inhibitors, such as derivatives of geldanamycin, by inducing Hsp40/Hsp70 expression [65,66]. Multiple studies have used the same types of drugs in vivo [65,67-70], providing evidence in immunoblots of tissue homogenates for Hsp40/Hsp70 induction in the CNS. However, it remains unclear to us whether Hsp40/Hsp70 can be robustly induced in CNS neurons in vivo because we have not found a study that provides histologic, or in situ, validation of induction of these genes in neurons, in the intact CNS. Still, it is promising that a number of geldanamycin derivatives, and other Hsp90 inhibitors, are available for further testing (Table 2) and one of these may be more effective in neurons.

Compound	Concentration Range	Reference
Geldanamycin	50 nM in culture	[75]
AT13387	10-1000 nMin culture	[76]
SNX5422	15-250 nMin culture	[77]
STA9090	1-250 nM in culture	[78]
Celastrol	0.2-10 µMin culture	[79]
BIIB021	0-250 nM in culture, 40 mg/kg in vivo	[80,81]
AUY922	10- 500 nM in culture, 20 mg/kg in vivo	[82,83]

Table 2: Hsp90 inhibitors currently under investigation

The resistance of neurons *in vivo*to induction of the heat-shock chaperone system may be due, at least partially, to the activity of an 8 kDa protein that is highly expressed in neurons [71], termed heat-shock factor binding protein 1 (HSBP1). Studies of the mechanism of action for HSBP1 in regulating heat-shock factor 1 (HSF1) activity are limited, but it appears that HSBP1 directly binds to a domain in HSF1 that is critical for the assembly of active trimeric complexes [72,73]. HSBP1 has also been described as a co-chaperone of Hsp70, which can be active in a negative feedback loop to disassemble and inactivate trimers of HSF1 [72]. A very recent study described the generation of HSBP1 knockout mice, demonstrating that homozygous null mice have defects in preimplantation embryonic development [74]. HSPB1 deficient ES cells show constitutive elevations in heat-shock chaperones. Thus, HSBP1 is a strong candidate to act as a negative regulator of the inducible chaperones and may account for the limited response of heat-shock chaperones in neurons*in vivo*.

Modification of proteostatic function may represent a good therapeutic target for patients at multiple stages of disease as early intervention could slow amyloid-induced aggregation of tau; whereas, late intervention could slow tau-induced aggregation of TDP-43 and other "by-stander" targets. Although there is rightfully increasing emphasis on early intervention in AD, we face an immediate future in which many patients have already aged beyond the window of opportunity for early intervention. Thus there is and will be a need for therapies that could modify disease in patients that have moderate to late-stage AD. If diminished proteostatic function and by-stander misfolding contributes to late-stage symptoms, drugs that restore proteostatic balance could prove to be effective in slowing the progression of disease.

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